THIOREDOXIN REDUCTASE AND GLUTATHIONE PEROXIDASE IN THE PREVENTION OF OXIDATIVE DAMAGE TO VASCULAR ENDOTHELIUM AND THE SKIN

BY

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This thesis is dedicated to my father, the late Dr Mike Lewin (1942 – 1989), who will forever be a source of pride and inspiration. Without him I would never have been shown the way, and I aspire to his inner peace every single day.

DECLARATION OF ORIGINALITY

I declare that this thesis is of my own composition, and the studies presented herein are the result of my own independent investigation. Work performed by others as part of collaborative studies are indicated in the text.

The work presented in this thesis has not been submitted for any other degree.

Michelle Helen Lewin

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ABSTRACT

Reactive oxygen species (ROS) contribute to the pathogenesis of a number of common and important diseases which include atherosclerosis and skin cancer. Selenium (Se) supplementation can protect skin and the endothelium from oxidative damage possibly by increasing the synthesis of antioxidant selenoproteins such as the family of glutathione peroxidases (GPX) and thioredoxin reductase (TR).

The relative importance of TR and GPX in protecting endothelial cells (EC) and skin cells from oxidative damage was studied using the EAhy926 and HaCaT cell lines as models of human endothelial cells and keratinocytes, respectively.

[^{75}Se]-labelled human umbilical vein EC (HUVEC) had a similar selenoprotein profile to EAhy926 cells. In HUVEC, human coronary artery EC (HCAEC), bovine aortic EC (BAEC) and EAhy926 cells, the expression of TR, cytoplasmic GPX (cyGPX) and phospholipid hydroperoxide GPX (PHGPX) was increased by incubating cells with increasing sodium selenite concentrations for 48 hr. Basal and Se-induced levels of these selenoproteins were similar in EAhy926 to HUVEC. BAEC differed considerably from HUVEC and EAhy926 cells in their selenoprotein expression. Therefore, EAhy926 cells appear to be a better model than BAEC for studies of selenoprotein function in humans.

In EAhy926 cells TR, cyGPX and PHGPX activities were induced 1.9-fold, 5.3-fold, and 2.6-fold respectively by sodium selenite supplementation (40 nM for 48 hr). Se-deficient EAhy926 cells were susceptible to oxidative damage by tertiary butyl hydroperoxide (t-BuOOH) and oxidised low density lipoprotein (oxLDL), as assessed using percentage retention of LDH. Cytotoxicity was attenuated ($p < 0.001$) by pre-incubation with 40 nM sodium selenite, a concentration which maximally induced TR and cyGPX.

Treatment of Se-deficient EAhy926 cells with gold thioglucose (GTG) (1 μM) significantly inhibited TR activity (74.8 % activity retained) ($p < 0.01$) but not cyGPX or PHGPX. Treated cells were more susceptible to oxidative damage by t-BuOOH ($p < 0.05$) or oxLDL ($p < 0.05$), suggesting that TR may provide antioxidant protection. Cells treated with 10 μM GTG showed inhibition of both TR and the GPXs (14.02 % TR activity ($p < 0.001$), 40.2 % cyGPX activity ($p < 0.001$), and 77.5 % PHGPX activity ($p < 0.01$) retained). Such cells were more susceptible to t-BuOOH toxicity than cells treated with 1 μM GTG ($p < 0.05$). Hence, both TR and the GPXs may be involved in the prevention of oxidative damage to human EC.

In HaCaT cells, expression of TR and cyGPX was optimally induced by incubation with sodium selenite concentrations of 10 nM and 100 nM (increased activities of 2.8-fold and

3.8-fold, respectively). Sodium selenite-treated HaCaT cells were significantly protected from oxidative damage mediated by UVB ($p < 0.001$) or menadione ($p < 0.01$).

Using UVB as the oxidative agent, loss of protection occurred at sodium selenite concentrations greater than 100 nM. At 1000 nM no protective effect of selenite was observed. There was an accompanying loss of cyGPX activity ($p < 0.05$), but not of TR or PHGPX expression. No loss of protection was demonstrated at the higher sodium selenite concentrations using menadione as oxidative stressor. The concentration of Se used for protection against UVB thus appears crucial.

Se-deficient HaCaT cells incubated with a GTG concentration (10 μM) that significantly inhibited TR activity (18.1 % activity retained) ($p < 0.001$) but not the GPXs were more susceptible to damage by menadione ($p < 0.05$), but showed no increase in susceptibility to UVB-mediated damage. Treatment with a GTG concentration (100 μM) which significantly inhibited both TR (3.18 % activity retained) ($p < 0.001$) and cyGPX activity (33.3 % activity retained) ($p < 0.001$) increased the susceptibility of HaCaT cells to UVB damage when compared to controls ($p < 0.01$). The data suggest that menadione, a model agent for UVB oxidative stress, may produce misleading results. TR appears to be important in protecting cells against damage mediated by menadione, but cyGPX to be more important in preventing damage caused by UVB. The two different oxidative stress agents may thus differ in their mechanism of toxicity.

TR expression regulated by Se supply and the redox state of the cell may affect cell growth. Changes in TR and cyGPX activity were investigated in human foetal (16-20 weeks gestation) and neonatal (1 day-15 weeks postnatal) liver cytosols. TR activity and concentration, and cyGPX activity in human foetal liver were approximately 3-fold greater than in neonatal liver. These human findings contrast markedly with results in the rat where TR and cyGPX activities increase throughout the foetal, newborn and adult stages. These results cast doubt on the rat as a model for studying cyGPX and TR in human development.

In conclusion, the data presented in this thesis suggest that both cyGPX and TR are important contributors to the antioxidant defence mechanisms of EAhy926 and HaCaT cells, and may therefore help to protect against atherogenesis and skin cancer formation respectively.

ABBREVIATIONS

A23187	calcium ionophore, A23187
AC	adenylate cyclase
ACh	acetylcholine
ANOVA	analysis of variance
AP-1	activator protein-1
apoB	apolipoprotein B
ARE's	AU-rich elements
ATP	adenosine triphoshate
AuTG	aurothioglucose
BAEC	bovine aortic endothelial cells
BCC	basal cell carcinoma
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BH_4	6(R)-5,6,7,8-tetrahydrobiopterin
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAM	cell adhesion molecule
cDNA	complementary deoxyribonucleic acid
cGMP	guanosine 3', 5'- cyclic monophosphate
CHD	coronary heart disease
Cu^{2+}	copper (II) ions
C-UCA	*cis*-urocanic acid
CV	coefficient of variation
CVD	cardiovascular disease
Cys	Cysteine residue
cyGPX	cytoplasmic glutathione peroxidase
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
EBSS	Earle's balanced salt solution
EBM-2	endothelial basal medium-2
EC	endothelial cell(s)
E.coli	Escherichia coli

EDHF	endothelium-derived hyperpolarizing factor
EDRF	endothelium-derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGM-2	endothelial growth medium-2
EGPX	extracellular glutathione peroxidase
eNOS	endothelial nitric oxide synthase
Erk	extracellular signal-regulated protein kinase
ET-1	endothelin-1
FABP	fatty acid-binding protein
FAD	flavin adenine dinucleotide
FADPH	flavin adenine dinucleotide phosphate
FBS	foetal bovine serum
Fe^{2+}	iron (II) ions
FMN	flavin mononucleotide
FCS	foetal calf serum
FGF-2	fibroblast growth factor-2
g	grams
g	centrifugal force
giGPX	gastrointestinal glutathione peroxidase
GM-CSF	granulocyte macrophage-CSF
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulphide (oxidised glutathione)
GST	glutathione-S-transferase
GTG	gold thioglucose
15-HPETE	15(S)-hydroperoxyeicosatetraenoic acid
HAT	hypoxanthine, aminopterin, thymidine
HBSS	Hank's balanced salt solution
HEPES	n-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HCAEC	human coronary artery endothelial cells
HCl	hydrochloric acid
Hcy	Homocysteine
HDL	high density lipoprotein
4-HNE	4-hydroxy-2-nonenal
HO	haem oxygenase
H_2O	water
H_2O_2	hydrogen peroxide

HOCl	hypochlorous acid
HPC	human placental cytosol
hr	hour
HUAEC	human umbilical artery endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule-1
IDI	iodothyronine deiodinase
IL	interleukin
IP_3	inositol (1, 4, 5) triphosphate
J/m^2	joules/square metre
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LC_{50}	lethal (50 %) concentration
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LOOH	lipid hydroperoxide
M199	medium 199
mA	milliamperes
MAPK	mitogen-activated protein kinases
MBq	mega Becquerel
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony stimulating factor
MDA	malondialdehyde
MED	minimal erythemal dose
MI	myocardial infarction
min	minute
mmLDL	minimally-modified low density lipoprotein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
mU	milli units
MPO	myeloperoxidase
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NBT	Nitro-Blue tetrazolium chloride
NEM	n-ethyl maleimide
NFκB	nuclear factor κB
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy

NMSC	non-melanoma skin cancer
NO	nitric oxide
NO_2^-	nitrite
NOS	nitric oxide synthase
NSB	non-specific binding
NSS	normal swine serum
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion
$O_2^{=}$	peroxide
ODC	ornithine decarboxylase
$OH^{\bullet}$	hydroxyl radical
OH^-	hydroxyl ion
ONOO-	peroxynitrite
oxLDL	oxidised low density lipoprotein
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCMB	para-chloro-methyl benzoate
PCOOH	phosphatidylcholine hydroperoxide
PDGF	platelet-derived growth factor
PDI	protein disulphide isomerase
PGE_2	prostaglandin E_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PHGPX	phospholipid hydroperoxide glutathione peroxidase
PK	primary keratinocytes
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
plGPX	plasma glutathione peroxidase
PMA	phorbol 12-mystrate 13-acetate
Prx	peroxiredoxin
PUFA	polyunsaturated fatty acid
PVDF	polyvinyldifluoride
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
RT	room temperature
SCC	squamous cell carcinoma

SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Se	selenium
sec	seconds
SECIS	selenocysteine insertion sequence
SeCys	selenocysteine
SD	standard deviation
SH-/ $(SH)_2$	thiol group
SMC	smooth muscle cell(s)
SOD	superoxide dismutase
SPS	selenoprotein synthetase
SSR	solar simulated radiation
TBARS	thiobarbituric acid reactive substances
t-BuOOH	tert-butylhydroperoxide
TEMED	NNN'N-tetramethylethylenediamine
TGFβ	transforming growth factor β
TNB	5'-thionitrobenzoic acid
TNFα	tumor necrosis factor-alpha
α-Toc	α-Tocopherol
t-PA	tissue plasminogen activator
TR	thioredoxin reductase
tRNA	transfer ribonucleic acid
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
Trx	thioredoxin
TXA_2	thromboxane A_2
UTR	mRNA 3' untranslated region
UV	ultraviolet radiation
V	volts
v/v	volume/volume
VCAM-1	vascular cell adhesion molecule-1
vWF	von Willebrand Factor
w/w	weight/weight
XO	xanthine oxidase

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1 OXIDATIVE STRESS

1.1.1 Introduction

Tissue damage mediated by reactive oxygen species (ROS) is believed to play an important pathogenic role in the development of various disorders and disease states including atherosclerosis and cancer (Halliwell, 1994; Halliwell and Gutteridge, 1999). Since the antioxidant capacity of tissues plays an important role in influencing their susceptibility to oxidative challenge, much research has focused on the assessment of antioxidant defence systems. This thesis examines the enzymatic antioxidant systems, in particular the thioredoxin and glutathione peroxidase systems, in relation to antioxidant protection of endothelial cell and skin cell models *in vitro*.

The utilization of oxygen by aerobic organisms generates large amounts of reactive oxygen species, leading to either physiological concentrations required for normal cell function, or excessive quantities, leading to oxidative stress. The term 'oxidative stress' denotes "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies, 1991). In principle, oxidative stress may result from diminished antioxidants or increased production of ROS. Correspondingly, the consequences of oxidative stress may be either adaptation (e.g. ischaemic pre-conditioning), damage or stimulation (e.g. of proliferation of vascular smooth muscle cells).

ROS can directly modify all major groups of biomolecules, DNA, lipids, and proteins, which results in mutations, strand breaks, aldehyde formation, lipid peroxidation, alterations in enzyme activities, and signal transduction pathways. Extracellular tissue components, including hyaluronic acid and collagen, are also vulnerable to injury by toxic oxidants. This may compromise the architectural integrity of tissues including the basement membrane of epithelia and blood vessels. The ubiquity of damage linked to ROS illustrates the vulnerable nature of cells and tissue to free radical attack.

There are many intracellular sources of ROS (figure 1.01). These include electron leakage from the mixed-function oxidase cytochromes P450 and b_5 in the endoplasmic reticulum, electron leakage from the mitochondrial electron transport chain, enzymatic reactions (e.g. xanthine oxidase), intracellular autooxidation of various compounds, arachidonic acid metabolism, transition metals (Fenton chemistry), and the activation of phagocytic cells (oxidative burst). ROS can also be formed in the skin directly or indirectly through endogenous photosensitization reactions.

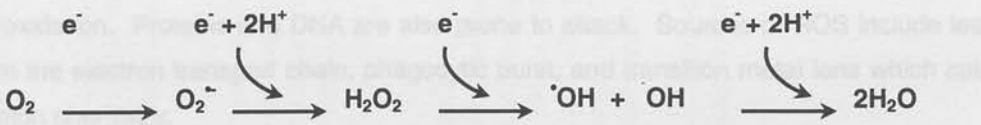
1.1.2 Reactive oxygen species (ROS)

A group of related terms is used in the scientific literature to refer to free radicals; these include oxyradicals and oxygen free radicals. The term reactive oxygen species (ROS) is generally preferred because singlet oxygen, hydrogen peroxide, hypochlorous acid and peroxide, and hydroperoxide and epoxide metabolites of endogenous lipids and xenobiotics contain chemically reactive oxygen-containing functional groups, but are not radicals and do not necessarily interact with tissues through radical reactions. Examples of ROS are listed in table 1.01. ROS include a number of chemically reactive molecules derived from oxygen. Some of these molecules are extremely reactive, such as the hydroxyl radical, while some are less reactive (superoxide and hydrogen peroxide). Free radicals and ROS can readily react with most biomolecules, starting a chain reaction of free radical formation. In order to stop this chain reaction, a newly formed radical must either react with another free radical, eliminating the unpaired electrons, or react with a free radical scavenger – a chain-breaking or primary antioxidant.

Table 1.01 Reactive oxygen species

Radicals	Non-radicals
Superoxide ($O_2^{\bullet -}$)	Hydrogen peroxide, (H_2O_2)
Hydroxyl radical ($OH^{\bullet}$)	Hypochlorous acid, (HOCl)
Peroxyl ($RO_2^{\bullet}$)	Ozone (O_3)
Alkoxyl ($RO^{\bullet}$)	Singlet oxygen ($^1O_2$)
Hydroperoxyl ($HO_2^{\bullet}$)	Peroxynitrite, ($ONOO^-$)

The step-wise reduction of molecular oxygen via 1-electron transfers produces superoxide, hydrogen peroxide and the hydroxyl radical, with the eventual four-electron reduction product water, which may be outlined as detailed below in reaction 1:



(reaction 1)

The cellular sources of ROS formation are summarised in figure 1.01, and their metabolism by cellular antioxidant systems is summarised in figure 1.02. Table 1.02 lists the most common intracellular forms of ROS, their main cellular sources of production, and the enzymatic antioxidant systems which scavenge these ROS molecules.

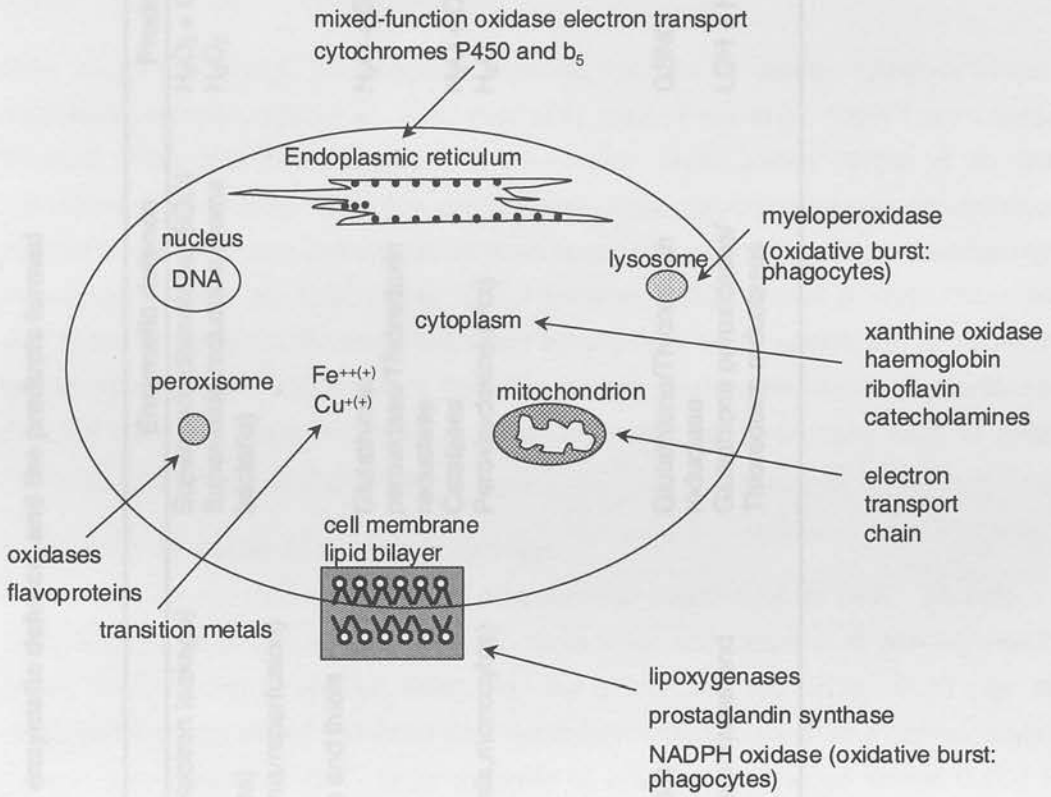


Figure 1.01 Cellular sources of reactive oxygen species. Many constituents of the cell are subject to oxidative attack. A frequent target is the cell membrane, resulting in lipid peroxidation. Proteins and DNA are also prone to attack. Sources of ROS include leakage from the electron transport chain, phagocytic burst, and transition metal ions which catalyse Fenton chemistry.

Table 1.02 The major ROS, their main cellular sources, enzymatic defence, and the products formed

ROS	Principal source(s)	Enzymatic defence	Product(s)
Superoxide ($O_2^{\bullet -}$)	Electron transport chain (electron leakage)	Superoxide dismutase (SOD)	$H_2O_2 + O_2$
		Superoxide reductase (some bacteria)	H_2O_2
	Oxidative burst (phagocytes)		
	Xanthine oxidase (ischaemia/reperfusion)		
	Flavoenzymes		
	Autooxidation of ascorbate and thiols		
Hydrogen peroxide (H_2O_2)	From $O_2^{\bullet -}$ via superoxide dismutase (SOD)	Glutathione peroxidase/Thioredoxin reductase	H_2O + GSSG
		Catalases	$H_2O + O_2$
	NADPH-oxidase (neutrophils,monocytes)	Peroxiredoxins (Prx)	H_2O
	Glucose oxidase		
	Xanthine oxidase		
Hydroxyl radical ($^{\bullet}OH$)	From $O_2^{\bullet -}$ and H_2O_2 via transition metals		
	Ionizing radiation		
Nitric oxide ($NO^{\bullet}$)	Nitric oxide synthases (NOS)	Glutathione/Thioredoxin reductase	GSNO
Lipid hydroperoxides (LOOH)	Lipid peroxidation of biomembranes and lipoproteins	Glutathione peroxidases/ Thioredoxin reductases	LOH + H_2O

(adapted from Nordberg *et al*, 2001)

1.1.3 Physiological functions of ROS

Neutrophils, eosinophils, and mononuclear phagocytes possess a flavoprotein NADPH oxidase system that converts O_2 to $O_2^{\bullet-}$. During phagocytosis, cells consume increased amounts of oxygen ('oxidative' or 'respiratory burst') to generate $O_2^{\bullet-}$, H_2O_2 (from $O_2^{\bullet-}$ via SOD), $OH^{\bullet}$, and hypochlorous acid (HOCl) (from H_2O_2 via myeloperoxidase) which are highly toxic to bacteria ingested by the phagocyte.

ROS also have crucial, advantageous physiological use in cellular functions including intracellular signalling (Go *et al.*, 1999; Pani *et al.*, 2000; Peus *et al.*, 1999; Thannickal and Fanburg, 2000) and redox regulation of transcription factor activity (Gupta *et al.*, 1999; Lakshiminarayanan *et al.*, 1998; Sun and Oberley, 1996). Several cytokines, growth factors, hormones, and neurotransmitters use ROS as secondary messengers in intracellular signal transduction (Bae *et al.*, 1997; Finkel, 1998; Pani *et al.*, 2000; Peus *et al.*, 1999; Thannickal and Fanburg, 2000). ROS can directly affect the structure and/or activities of all sulphydryl-containing molecules by oxidation of their thiol groups. Such redox regulation affects many proteins which are important in signal transduction and carcinogenesis, such as protein kinase C, collagenase, and tyrosine.

1.1.4 ROS as cause of oxidative damage

ROS are mutagenic (Marnett, 2000), due to chemical modification of DNA. Alterations to DNA, such as DNA cleavage, DNA-protein cross links, and oxidation of purine/pyrimidine bases (Halliwell and Gutteridge, 1999), are due to reactions with ROS. ROS may also damage DNA indirectly by activating Ca^{2+}-dependent endonucleases as a consequence of rises in intracellular free Ca^{2+}, or by a variety of aldehyde derivatives formed during the oxidation of lipids, proteins or amino acids.

Polyunsaturated fatty acids (PUFA) of membranes and lipoproteins are prime targets for free radical attacks. Generation of peroxyl and alkoxyl radicals, aldehydes (especially 4-hydroxy-2-nonenal; 4-HNE), and other products of lipid peroxidation in membranes and lipoproteins can cause severe damage to the proteins present by reacting with thiol groups of GSH or cysteine in proteins, altering their functions. Aldehydes can also react with amino groups.

ROS react with several amino acid residues *in vitro*, generating modified and less active enzymes, cross-linked, denatured, or inactive proteins (Halliwell and Gutteridge, 1999). Damage to proteins can occur by direct attack by ROS, or by secondary damage by lipid peroxidation end-products such as malondialdehyde (MDA) and 4-HNE. General antioxidant systems such as Trx, Grx, or GSH, or specific systems, such as methionine sulphoxide reductase, maintain the protection of proteins from such attack and modification.

1.1.5 Cellular antioxidant systems

The cellular antioxidant systems can be divided into two major groups, enzymatic and nonenzymatic. The antioxidant enzyme systems of the cell are illustrated in figure 1.02.

a) Enzymatic antioxidants

i) Superoxide dismutases (SOD)

Superoxide dismutase (SOD) catalyses the dismutation of $O_2^{\bullet-}$ to oxygen and H_2O_2. Human tissue contains three isoenzymes of SOD: an 80kDa tetrameric mitochondrial manganese-containing SOD (Mn-SOD), a 32-kDa dimeric cytosolic copper-zinc SOD (Cu/Zn-SOD) and a larger Cu/Zn-SOD, distinct from the cytosolic type, is found extracellularly.

ii) Catalases

Catalases of mammalian origin are mainly haem-containing enzymes of four subunits, each with a ferric haem group and NADPH bound. The predominant subcellular localization is in peroxisomes, where the enzyme catalyzes the dismutation of H_2O_2 to water and molecular oxygen. Mammalian catalases also have some peroxidase-like activity.

iii) Peroxiredoxins (Prx)

The thioredoxin peroxidases belong to a conserved family of proteins, the peroxiredoxins (Prx). Prx catalyze reduction of peroxides, e.g. H_2O_2 and alkyl hydroperoxides (Berggren *et al.*, 2001; Chae *et al.*, 1999) using Trx as their source of reducing equivalents in mammalian cells (Chae *et al.*, 1999). Prx I and II are abundant in the cytosol (Kim *et al.*, 2001a).

iv) Glutathione peroxidases (GPX)

There are four different selenocysteine-containing GPXs in mammalian cells. The GPX family of enzymes is described in detail in section 1.2.7.

v) Glutathione S-transferase (GST)

Many xenobiotics are metabolised by conjugation with GSH, catalysed by the glutathione S-transferase (GST) enzymes. Eukaryotes have multiple cytosolic and membrane-bound GST isoenzymes, each with distinct substrate specificities (Halliwell and Gutteridge, 1999). Some GSTs metabolise cytotoxic aldehyde products of lipid peroxidation, or show GPX-like activity, reducing organic peroxides (but not H_2O_2).

vi) The mammalian thioredoxin system (Trx-TR)

The Trx-TR system is described in detail in section 1.2.7.

vii) Haem-oxygenase-1 (hsp32/HO-1)

Haem-oxygenase (HO) is the rate-limiting enzyme in haem catabolism, which degrades haem to carbon monoxide and biliverdin with the release of iron (Otterbein and Choi, 2000). Iron is then sequestered by ferritin, preventing ROS production. The HO-1 isoform is a stress protein that is induced by oxidative stress (Clark *et al.*, 2000; Otterbein and Choi, 2000; Vile *et al.*, 1994).

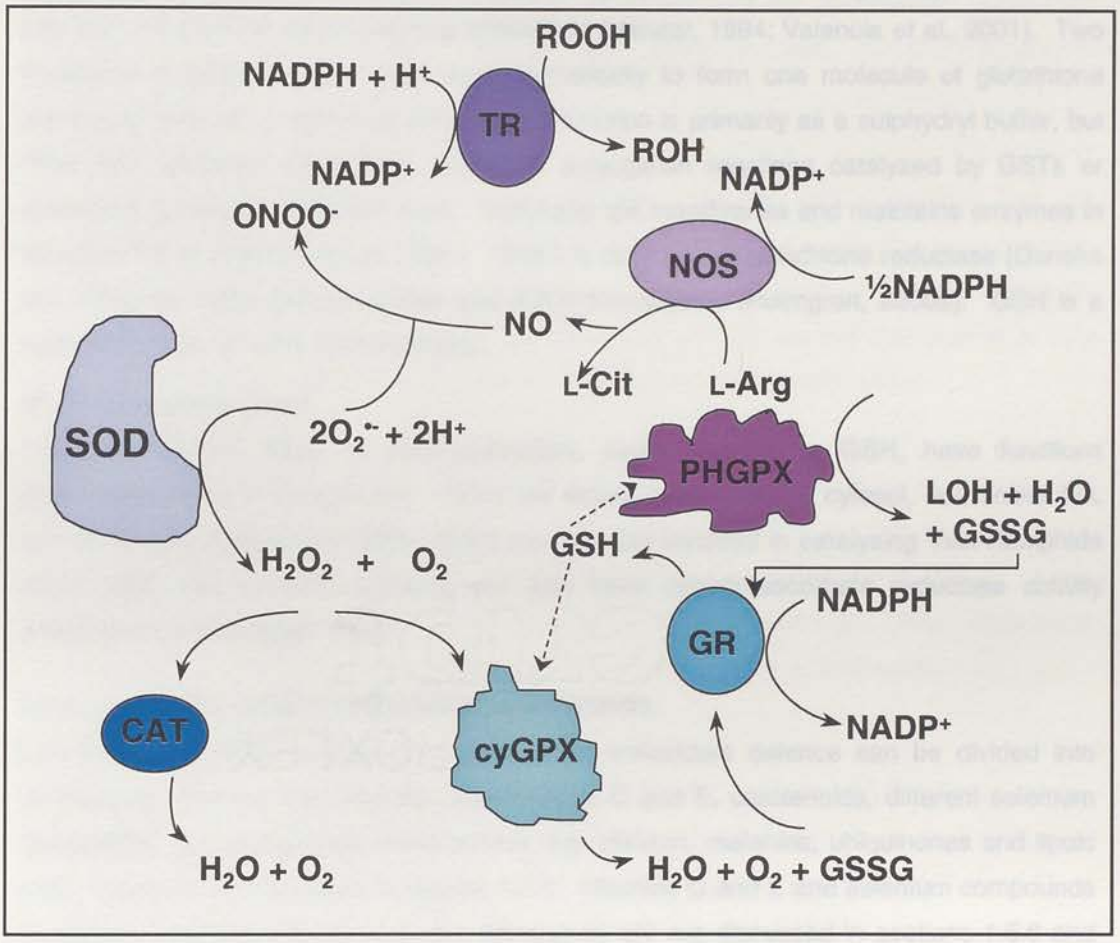


Figure 1.02 The endogenous antioxidant enzyme systems of the cell. CAT, catalase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione, GSSG, glutathione disulphide; H_2O_2, hydrogen peroxide; L-Arg, L-Arginine; L-Cit, L-Citrulline; $O_2^{\bullet-}$, superoxide anion; $ONNO^-$, peroxynitrite; NO, nitric oxide; NOS, nitric oxide synthase; TR, thioredoxin reductase. Also of importance are the peroxiredoxins (not included in the diagram), which can reduce H_2O_2. Non-enzymatic antioxidants are also importance for defence against oxidative damage. Low molecular weight compounds considered to be antioxidants of biological importance include vitamins C and E, different selenium compounds, lipoic acid, and ubiquinones.

b) Non-enzymatic antioxidants

i) Glutathione (GSH)

Glutathione (GSH), an endogenous tripeptide (glutamate, cysteine, glycine), is the most abundant intracellular thiol-containing antioxidant (Meister, 1994; Valencia *et al.*, 2001). Two molecules of reduced GSH react non-enzymatically to form one molecule of glutathione disulphide (oxidised glutathione) (GSSG). Its function is primarily as a sulphydryl buffer, but GSH also detoxifies compounds either via conjugation reactions catalyzed by GSTs or directly by donating a hydrogen atom. It protects cell membranes and maintains enzymes in bioactive forms (Valencia *et al.*, 2001). GSSG is recycled by glutathione reductase (Deneke and Fanburg, 1989) and by human and *E.coli* thioredoxins (Holmgren, 2000a). GSH is a hydrogen donor for GPX and ascorbate.

ii) Glutaredoxins (Grx)

The glutaredoxins (Grx), or thioltransferases, closely related to GSH, have functions overlapping those of thioredoxins. Grx's are largely located in the cytosol, but, unlike Trx, can be directly reduced by GSH. Grx's are possibly involved in catalysing thiol-disulphide interchange with cytosolic proteins, but also have dehydroascorbate reductase activity (Halliwell and Gutteridge, 1999).

Low molecular weight antioxidant compounds

Low molecular weight compounds important in antioxidant defence can be divided into compounds obtained from the diet, e.g. vitamins C and E, carotenoids, different selenium compounds, and compounds made *in vivo*, e.g. bilirubin, melanins, ubiquinones and lipoic acid. Melanins are discussed in section 1.7.1. Vitamins C and E and selenium compounds in relation to atherosclerosis and skin damage by UV are discussed in sections 1.5.6 and 1.6, and 1.10 and 1.11.4, respectively.

i) α-tocopherol

α-tocopherol (Vitamin E) is a lipophilic, chain-breaking antioxidant. Attached to the hydrophobic structure of α-tocopherol is an –OH group whose hydrogen atom is easily donated (Halliwell and Gutteridge, 1999). Peroxyl and alkoxyl radicals generated during lipid peroxidation combine with α-tocopherol instead of the PUFA, terminating the chain reaction. The α-tocopherol is converted to the α-tocopheryl radical which is poorly reactive and unable to attack PUFA. Vitamin C is involved in regenerating α-tocopherol from the α-tocopheryl radical, as are ubiquinol and GSH to a lesser extent (Halliwell and Gutteridge, 1999).

ii) Ascorbate

Ascorbic acid (vitamin C) is a water-soluble vitamin that cannot be manufactured in the human body, and so needs to be present in the diet. It is the major antioxidant in protection of cells and human plasma from oxidative stress. Ascorbic acid has two ionisable –OH

groups, and is a reducing agent (Halliwell and Gutteridge, 1999). Donation of one electron by ascorbate gives the ascorbyl radical which can be oxidised to dehydroascorbate (DHA). The ascorbyl radical is relatively unreactive, and DHA is unstable and breaks down rapidly. Reduction of the ascorbyl radical and DHA is carried out by mammalian thioredoxin reductase (May *et al.*, 1998a; May *et al.*, 1997) as well as GSH-dependent dehydroascorbate reductase.

iii) Carotenoids

The carotenoids are a group of coloured pigments (orange, red or yellow) that are widespread in plant tissues, and include lycopene, β-carotene, α-carotene, and lutein. Carotenoids are the hydrophobic precursors of the fat-soluble vitamin A (retinol). Radicals can react with carotenoids by electron transfer, since the carotenoid molecule is a long alternating single- and double-bond structure, allowing electron delocalisation.

iv) Ubiquinone

Coenzyme Q (ubiquinone) forms part of the electron transport chain in mammals, and functions as an antioxidant in mitochondria, and to a lesser extent in the plasma and plasma membranes. The coenzyme accepts one electron to form a semiquinone radical, or two electrons to form a fully-reduced ubiquinol form (Halliwell and Gutteridge, 1999). Ubiquinine also accepts electrons from reduced flavoproteins and β-oxidation of fatty acids.

1.1.6 Antioxidant synergy

Antioxidant defences operate as a balanced and coordinated system, and each relies on the action of the others. Antioxidant enzymes cooperate with antioxidants to defend against oxidative stress-induced cellular damage. The survival time of rats exposed to pure O_2 is increased by ~ 70 % when liposomes containing both catalase and SOD are intravenously injected before and during O_2 exposure; liposomes containing catalase or SOD alone are much less protective (Turrens *et al.*, 1984). Increased Cu/Zn-SOD levels in stable transfectants results in sensitization to oxidative DNA damage, which can be counteracted or overcome by co-transfection with GPX or catalase (Amstad *et al.*, 1994).

Ascorbate and GSH interact *in vivo* (Meister, 1994). Treatment of newborn rats with buthionine sulphoximine (BSO), a GSH-depleting agent, results in marked depletion of tissue ascorbate in liver, kidney, eye, lung and brain. When ascorbate is given to newborn rats, the levels of tissue and mitochondrial GSH increase, suggesting that ascorbate can spare GSH.

Some vitamins show synergistic effect if they exist simultaneously as antioxidants (May *et al.*, 1998b; Nègre-Salvayre *et al.*, 1995; Pryor, 2000b). This sort of synergy may be expressed as the beneficial effect on blood lipids. A full complement of multi-vitamins and multi-minerals taken orally can favourably modify lipid profiles and plasma peroxide levels and reduce the risk of cardiovascular diseases (Morcos, 1999; Morcos and Tomita, 1996).

1.2 Selenium

1.2.1 Introduction

Since the discovery that selenium (Se) is essential to animals and that it is necessary for the prevention of various disease states, research on the metabolism and the effects of this element has grown rapidly. Discovered by Berzelius in 1817, it was the 1950s before Se was first appreciated for its practical role in prevention of vascular disorders and nutritional myopathies in livestock. The true nutritional significance of this element was brought to the fore when, in 1973, it was identified as the biologically active component of the enzyme cytosolic glutathione peroxidase (GPX) (Rotruck *et al.*, 1973), preceded by the perception of its fundamental role in the prevention of liver necrosis in vitamin E-deficient rats in 1957 (Schwartz and Foltz, 1957). Reports highlighting the importance of Se in human nutrition appeared in 1979 describing the Se-responsive cardiomyopathy of Keshan disease in China (Group, 1979). Since then a considerable amount of subsequent research has demonstrated that Se is essential to human nutrition through the expression of a wide, and growing range of selenoproteins which have multiple and diverse roles.

1.2.2 The chemistry of selenium

Se is an essential element for the growth of humans and animals. However, its role in the mammalian diet is somewhat ambivalent since at lower concentrations it is essential for growth, but it exhibits toxicological properties at higher doses. Se lies between sulphur and tellurium in Group VI of the periodic table, with an atomic weight of 78.96. It is classed as a metalloid by virtue of sharing properties of both metal and non-metals. The element exists naturally in a range of oxidation states, combining with other elements to form inorganic selenides, selenites and selenates. Se also forms organic selenoamino acids, which include selenocysteine and selenomethionine. Despite sharing some physical and chemical properties, Se and sulphur are not interchangeable in biological systems (Foster and Sumar, 1997).

1.2.3 Se Metabolism and Bioavailability

a) Introduction

The metabolism of Se in animals and humans, i.e. its absorption, transport, distribution, excretion, retention and transformation to the active selenide form is dependent on its chemical form and the overall Se status of the individual. Animals normally receive dietary Se as organic selenoamino acids such as selenomethionine and selenocysteine and as methylated and non-methylated Se, though inorganic forms, such as sodium selenite and sodium selenate, are used in experimental diets and as supplements. Se is contained in association with tissue proteins throughout the body. Proteins that incorporate Se endogenously in stoichiometric amounts are referred to as selenoproteins and are

metabolically active, whereas proteins that bind Se non-specifically are termed Se-containing proteins.

The only reductive metabolic pathway for Se that has been well characterized is that based on the metabolism of most forms of Se to selenite and further reduction to selenide. Figure 1.03 shows a hypothetical scheme of Se metabolism adapted from Ip (Ip, 1998).

b) Absorption

Se is readily absorbed from the intestine and extracted rapidly by the liver and erythrocytes. Under normal feeding conditions absorption is not the limiting factor to Se bioavailability (Mutanen, 1986). L-Selenomethionine and L-methionine share the same active transport mechanism (McConnell and Cho, 1965), and absorption of inorganic forms such as selenite and selenate is via a passive mechanism (Thomson, 1998). Virtually complete absorption occurs when Se is supplied as selenomethionine (Swanson *et al.*, 1991) and other forms are generally well absorbed. Although different forms of Se are absorbed through different mechanisms, most forms are absorbed through the duodenum (Thomson, 1998). Absorption is unaffected by Se status which would suggest that no homeostatic regulation of absorption exists. The bioavailability of Se can be dependent on the levels of methionine and vitamins in the diet, total protein content, restricted diet intake, and the presence of heavy metals.

c) Transport

Se is transported in plasma bound to protein. Selenoprotein P (SelP) has been identified in the plasma of both rats and humans (Burk and Hill, 1994), accounting for over half of the Se content of mammalian plasma. It has been proposed to have a Se transport function (Motsenbocker and Tappel, 1982), and new data from a selP knockout mouse confirms this (Hill *et al.*, 2002).

d) Metabolism and distribution

The metabolism of Se is a complex process and varies according to the particular chemical form ingested (figure 1.03). Most forms of Se are metabolized to selenite, further reduced to selenides, or both. The bioavailability of these forms differs however. Se retention is greater when introduced as selenomethionine, which relates to non-specific incorporation of selenomethionine into proteins via the usual method of methionine incorporation (Nève, 1998), especially into skeletal muscle protein. However, selenomethionine in place of methionine confers no additional catalytic activity within a protein (Rayman, 2000; Waschulewski and Sunde, 1988).

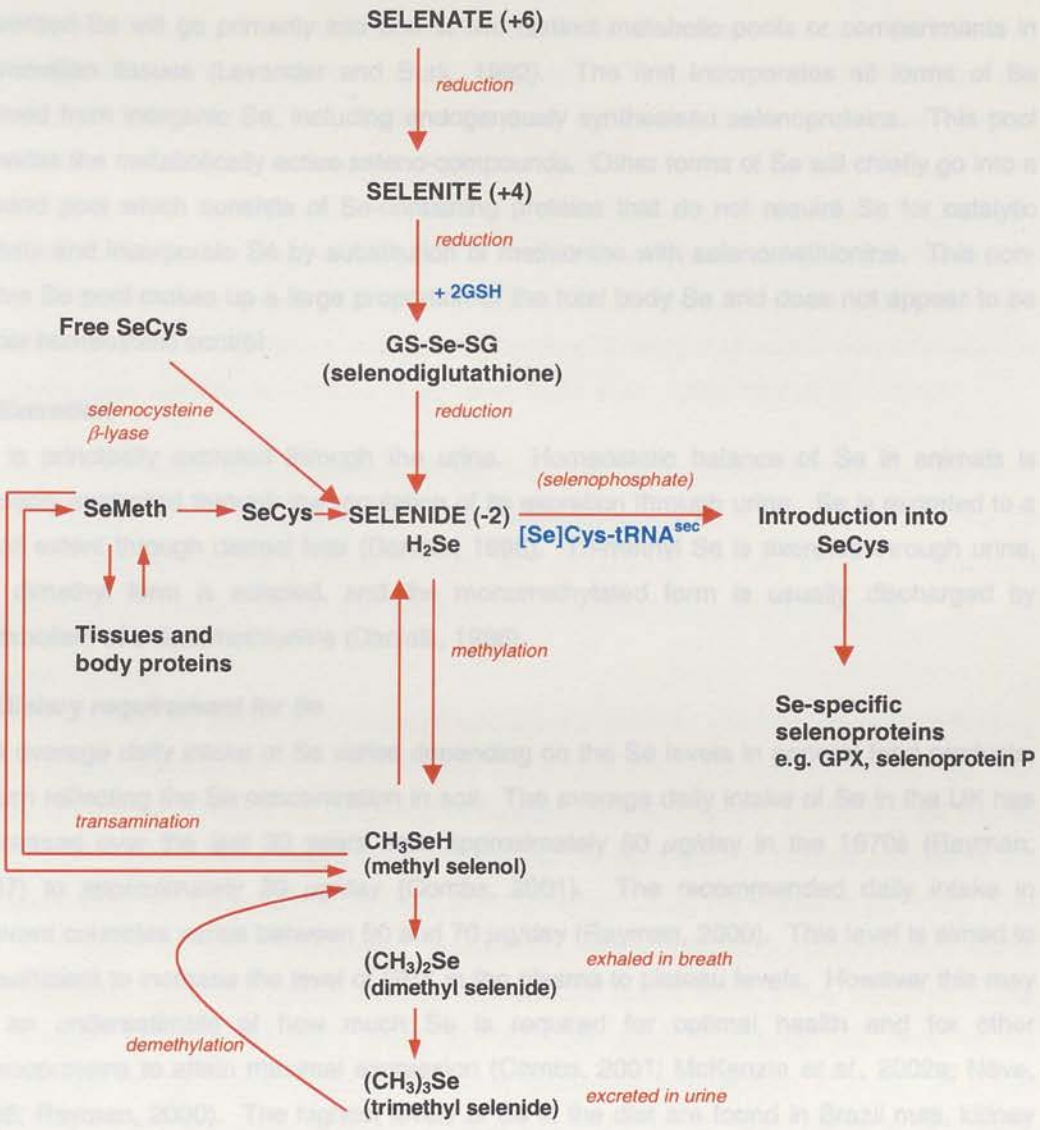


Figure 1.03 A diagram illustrating the pathways of Se metabolism. Adapted from Ip (Ip, 1998). The figure shows the pathway through which most forms of Se are metabolised and the precursors for the synthesis of different forms of selenoproteins and Se-containing proteins. Selenide is a precursor of selenophosphate, the universal Se donor *in vivo*. Selenomethionine (SeMeth) is not directly available for utilisation in specific Se pathways until it has been catabolized to selenocysteine (SeCys) by trans-sulphuration. The resulting SeCys does not accumulate; instead it is reduced to selenide to enable its Se to be made available for selenoprotein synthesis. Trimethyl selenide in urine can be sequentially demethylated to methyl selenol, which is then returned to the selenide pool where it may be used in selenoprotein manufacture.

Absorbed Se will go primarily into one of two distinct metabolic pools or compartments in mammalian tissues (Levander and Burk, 1992). The first incorporates all forms of Se derived from inorganic Se, including endogenously synthesised selenoproteins. This pool provides the metabolically active seleno-compounds. Other forms of Se will chiefly go into a second pool which consists of Se-containing proteins that do not require Se for catalytic activity and incorporate Se by substitution of methionine with selenomethionine. This non-active Se pool makes up a large proportion of the total body Se and does not appear to be under homeostatic control.

e) Excretion

Se is principally excreted through the urine. Homeostatic balance of Se in animals is primarily controlled through the regulation of its excretion through urine. Se is excreted to a small extent through dermal loss (Daniels, 1996). Tri-methyl Se is excreted through urine, the dimethyl form is exhaled, and the monomethylated form is usually discharged by metabolism of selenomethionine (Daniels, 1996).

f) Dietary requirement for Se

The average daily intake of Se varies depending on the Se levels in general food products, in turn reflecting the Se concentration in soil. The average daily intake of Se in the UK has decreased over the last 30 years from approximately 60 μg/day in the 1970s (Rayman, 1997) to approximately 30 μg/day (Combs, 2001). The recommended daily intake in different countries varies between 50 and 70 μg/day (Rayman, 2000). This level is aimed to be sufficient to increase the level of GPX in the plasma to plateau levels. However this may be an underestimate of how much Se is required for optimal health and for other selenoproteins to attain maximal expression (Combs, 2001; McKenzie *et al.*, 2002a; Nève, 1995; Rayman, 2000). The highest levels of Se in the diet are found in Brazil nuts, kidney and fish, whereas levels in dairy products are much lower. In response to the requirement for Se to sustain human health, several types of Se-enriched foods have been developed in recent years, including Se-fortified cereal, margarine, salt, and beverages, as well as foods grown in Se-enriched soil (Combs, 2001). Se supplements are sold in the form of both selenomethionine and sodium selenite (Schrauzer, 2001; Whanger, 2002), but the recommended dietary daily allowance is irrespective of the chemical compound consumed (Brigelius-Flohé *et al.*, 1995).

1.2.4 Se-Related Human Diseases and Se Toxicity

Health conditions associated with selenium deficiency

Low dietary Se intake has been implicated in the development of numerous health disorders in humans. These include, Kashin-Beck disease, cancer, cardiovascular disease (including Keshan disease), atherosclerosis (detailed in section 1.6), muscular dystrophy, malaria, arthritis, alopecia areata, pregnancy hypertension syndrome, altered immune function, male

infertility, spontaneous abortions, pre-eclampsia, and AIDS (Baum *et al.*, 1997; Combs, 2001; Combs and Clark, 1999; Foster and Sumar, 1997; Levander, 1987; Robinson and Thomson, 1983; Ximin *et al.*, 1998). Patients administered total parenteral nutrition (intravenous feeding) or enteral nutrition (tube feeding) long-term without Se-supplementation in their formulation run the risk of Se deficiency (Hatfield, 2001; Levander, 1987) which has been linked with myopathy (Brown *et al.*, 1986; Kien and Ganther, 1983) and cardiomyopathy (Fleming *et al.*, 1982; Johnson *et al.*, 1981). The relationship between the symptoms and Se-deficiency is supported by data showing that supplementation with Se alleviates many symptoms (Abrams *et al.*, 1992; Saito *et al.*, 1998; Yagi *et al.*, 1996b).

Among the diseases associated with severe endemic Se deficiency in humans, the most convincing data for an etiological role of Se-deficiency exists for Keshan and Kashin-Beck diseases. The occurrence of each of these two diseases is in rural areas of China and Russia (Eastern Siberia) where food systems have an exceedingly low Se supply.

Keshan disease

Keshan disease is a multifocal myocarditis occurring in children and females of child-bearing age. Supplementation of the diets of over one million rural-dwelling individuals with Se-enriched salt has reduced the incidence of Keshan disease, thereby confirming the causal relationship between Se-deficiency and the disease (Hatfield, 2001). Certain features of the disease cannot be explained by Se-deficiency alone, including the seasonal variation. The low Se status may be an underlying, pre-disposing factor, but Se supplementation works prophylactically only; Se cannot reverse the cardiac failure once it has occurred. The involvement of other cofactors, such as the viral agent coxsackie B3 virus in the development of the cardiomyopathy has been suggested from studies in mice. In a Se-deficient host the coxsackie virus can mutate into a cardiotoxic form (Beck *et al.*, 1995). Coxsackie virus has been isolated from tissues of some Keshan disease victims (Li *et al.*, 2000). Coxsackie virus recovered from cyGPX knockout mice undergoes mutation, and such knockout mice develop myocarditis, while virus from wild-type mice is unchanged, and wild-type mice are resistant to myocarditis (Beck *et al.*, 1998).

Kashin-Beck disease

Kashin-Beck disease is a Se-responsive endemic osteoarthropy (deforming arthritis), affecting preadolescent and adolescent children (Combs, 2001; Levander, 1987). The principal pathological feature of this disease is the necrosis of the chondrocytes during bone growth. The Se-enrichment of table salt for a Chinese population of 5 million individuals resulted in dramatic decreases in new cases, with the incidence rate decreasing to almost zero in communities which were formerly endemic (Hatfield, 2001). However, the complete absence of this disease in Se- and iodine-deficient central Africa suggests multiple

etiological factors, which may include poisoning by mycotoxins, fulvic acids in drinking water, and nutritional mineral imbalances (Combs, 2001; Hatfield, 2001; Levander, 1987; Tomlinson, 1999).

Cancer

Evidence from animal experiments and epidemiological studies suggests that Se can diminish cancer risk (Combs and Clark, 1999). Epidemiological studies have provided evidence of an inverse relation between Se intake (or Se status) and cancer risk (Clark *et al.*, 1991; Combs and Clark, 1999; Fex *et al.*, 1987; Knekt *et al.*, 1998; Salonen *et al.*, 1984; Virtamo *et al.*, 1987). The potency of Se is illustrated by a meta-analysis of the combined data from several studies (Ip, 1998) comparing the significance of serum Se, retinol, β-carotene, and vitamin E with respect to cancer risk (Comstock *et al.*, 1992). Se was the factor with the most consistently protective effect.

The most convincing evidence for the inverse relationship between Se and certain forms of cancer to date comes from a randomised double blind, placebo-controlled intervention study carried out by Clark *et al.* (Clark *et al.*, 1996; Clark *et al.*, 1998). 1312 subjects with a history of at least 2 skin cancers, from the (Se-poor) South-Eastern region of the USA received either 200 μg Se (as Se yeast) or a placebo daily. The results reported in 1996, 13 years after the initiation of the trial, showed no effect on the primary endpoint of non-melanoma skin cancers. However, those receiving Se showed secondary endpoint effects of 50 % lower total cancer mortality ($p = 0.002$) and 37 % lower total cancer incidence ($p = 0.001$) with 63 % fewer cancers of the prostate, 58 % fewer cancers of the colon, and 46 % fewer cancers of the lung. This study has however been criticised (Colditz, 1996; Ip, 1998; Parker, 1997; Pocock and Hughes, 1990).

The PRECISE (Prevention of Cancer by Intervention with Selenium) trial is the most recent Se supplementation trial, and has been designed to confirm/refute the results of Clark's trial. It has recently commenced with cohorts in the UK, Denmark, Sweden, Finland and the USA. A total of 33,000 patients will receive 100, 200 or 300 μg of Se a day, the trial will last for 5 years and will study the mortality rate in all of the groups.

In addition, the US National Cancer Institute is to fund a 12 year study, SELECT (Selenium and Vitamin E Cancer Prevention Trial), in which 32,000 men will be recruited to determine the effect of Se supplementation (200 μg per day as selenomethionine) and vitamin E on the risk of prostate cancer.

Another large scale supplementation trial was started in 1994 in France (SUVIMAX trial). The trial is to be carried out for 8 years on 15,000 individuals to investigate whether Se and zinc supplementation reduces the incidence of cancer and heart disease. The results will be published in 2003.

1.2.5 Selenium toxicity

Although Se is an essential element, it can become toxic at high concentrations. Se toxicity is dependent on the chemical form of Se administered, animal species, and quality of dietary protein (Spallholz, 1994).

The toxicity of Se at high concentration is thought to be due to its prooxidant ability to catalyse oxidation of thiols and generate $O_2^{\bullet-}$, H_2O_2 and other ROS (Spallholz, 1994; Spallholz, 1997). Cytotoxic Se compounds include selenite and selenocystine, which can generate $O_2^{\bullet-}$ and lead to an increase in oxidative DNA damage in the cell leading to apoptosis (Stewart *et al.*, 1999). Selenite can also react with GSH to produce selenodiglutathione, a very reactive compound which has apoptosis-inducing and carcinostatic properties (Ip, 1998; Spallholz, 1994). Mitochondria appear to be the main target of Se-induced oxidative stress (Spallholz, 2001). Documented cytotoxic effects of selenite (1 – 5 μM) include cytoplasmic vacuolisation, cell detachment, and membrane damage (Ip, 1998). Selenomethionine is not cytotoxic to cells even at high concentrations since it does not undergo redoxing-cycling and does not produce $O_2^{\bullet-}$ (Stewart *et al.*, 1999).

The tolerable upper intake level for Se, i.e. the highest level of daily intake that is likely to pose no risk of adverse health effects, was set at 400 μg/day for adults in the year 2000 (Hatfield, 2001). The 'Lowest Adverse Effect Level', defined as the 'average daily selenium intake causing individuals within a population to develop overt signs of toxicity' is thought to be in the order of 1540 ± 653 μg/day (Schrauzer, 2001).

1.2.6 Selenoprotein synthesis

Se is incorporated specifically into selenoproteins as selenocysteine (SeCys) residues through a co-translational event directed by the UGA codon (Heider *et al.*, 1992). This UGA codon has a dual role in the genetic code; in selenoprotein synthesis the UGA codon indicates the site of SeCys incorporation, whilst the alternative interpretation of the UGA codon recognises it as a termination codon.

The mechanism for the synthesis of selenoproteins was characterised in *E. coli* mutants (Bermano *et al.*, 1996a; Heider *et al.*, 1992). The synthesis of SeCys and its insertion into specific selenoproteins in prokaryotes involves the products of four genes (*selA*, *selB*, *selC*, and *selD)* (Allan *et al.*, 1999). The products are; a selenocysteine-specific tRNA species ($tRNA^{Sec}$) (*selC)* which carries the anticodon for UGA, the enzymes, selenocysteine synthase (*selA*) and selenophosphate synthetase (*selD*) that are essential for the formation of selenocysteine- $tRNA^{Sec}$ from seryl-$tRNA^{Sec}$ and the elongation factor that specifically recognises the selenocysteine-tRNA (*selB).* The *selB* product is similar to the elongation factor-Tu (Hatfield, 2001) which transports amino acid tRNAs to the ribosome. The major steps of selenocysteine insertion are illustrated in figure 1.04.

In eukaryotes, the process of selenoprotein synthesis differs in some features from the mechanism in *E.coli.* At least two forms of the $tRNA^{Sec}$ have been isolated in eukaryotes and both contain the UGA anticodon which is functional in *E. coli* (Kollmus *et al.*, 1996; Low and Berry, 1996). Like bacterial $tRNA^{Sec}$, in eukaryotes $tRNA^{Sec}$ is esterified with serine and is subsequently converted to seryl-$tRNA^{sec}$.

The nature of the mRNA selenocysteine insertion sequence (SECIS) elements which are responsible for the recognition of the UGA as a selenocysteine insertion codon differs between prokaryotes and eukaryotes (Kollmus *et al.*, 1996). In prokaryotes the SECIS stem-loop structure is immediately downstream (3') from the UGA codon in the open reading frame. In contrast eukaryotic SECIS elements are located in the 3'-untranslated region (UTR) of the mRNA (Berry *et al.*, 1993). The SECIS sequences vary for different selenoproteins, but serve a common purpose. Differences in stem-loop function may permit the translation machinery of the cell to express some selenoproteins selectively in preference over others (Berry *et al.*, 1993).

Two selenophosphate synthetases have been identified in eukaryotes, *Sps1* and *Sps2* (see section 1.2.7). In addition to *Sps 1* and *2*, two proteins have been identified in eukaryotes that carry out the functions equivalent to *SelB*. These proteins, one of which binds to the SECIS sequence (SBP2) and a second that is a selenocysteyl-$tRNA^{[Ser]Sec}$-specific elongation factor (eEFSec), allow the translation of UGA as SeCys instead of a termination codon.

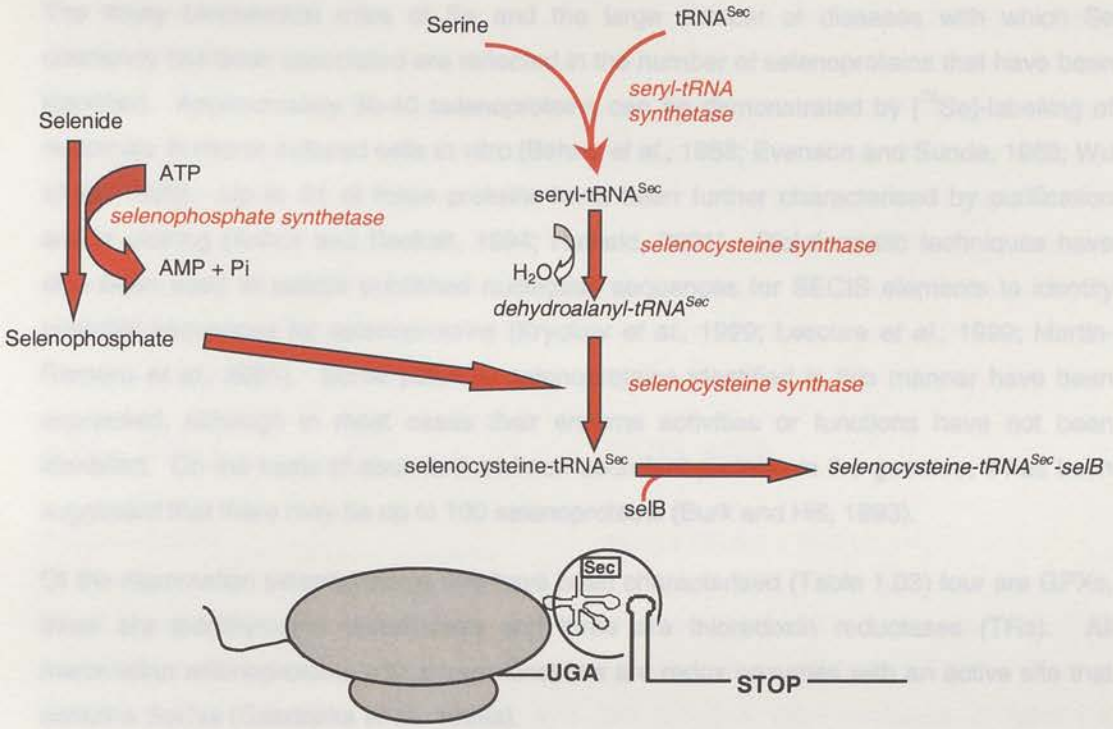


Figure 1.04 A proposed model of the incorporation of selenocysteine into selenoproteins in prokaryotes. The selenocysteine specific tRNA ($tRNA^{Sec}$) is initially esterified with L-serine, catalysed by seryl-tRNA synthetase. Once charged with L -serine the seryl-$tRNA^{Sec}$ can bind to selenocysteine synthase. Selenocysteine synthase is a pyridoxyl phosphate- containing enzyme which catalyses the conversion of seryl-$tRNA^{Sec}$ to selenocysteyl-$tRNA^{Sec}$. The initial step involves elimination of a water molecule to form a dehydroalanyl-$tRNA^{Sec}$ intermediate. The second step involves the addition of a reactive selenium derivative; monoselenophosphate acts as the Se donor. It is produced from selenide and ATP by selenophosphate synthetase. The selenocysteyl-$tRNA^{Sec}$ (sec-$tRNA^{Sec}$) is then released from the selenocysteine synthase and binds an elongation factor selB which delivers the sec-$tRNA^{Sec}$ complex to a stem loop structure on the mRNA. In order to recognize the UGA codon as an insertion sequence for selenocysteine as opposed to a stop codon the stem loop structure has a specific sequence downstream from the UGA codon. The stem loop structure binds the *selB*-$tRNA^{Sec}$-selenocysteine complex aiding its interaction with the UGA codon and the subsequent incorporation of selenocysteine into the protein. SelB is represented by the circle, with the selenocysteyl-$tRNA^{Sec}$, the stem-loop and the ribosome approaching the UGA codon.

1.2.7 Mammalian selenoproteins and Se-containing proteins

The many biochemical roles of Se and the large number of diseases with which Se deficiency has been associated are reflected in the number of selenoproteins that have been identified. Approximately 30-40 selenoproteins can be demonstrated by [^{75}Se]-labelling of mammals *in vivo* or cultured cells *in vitro* (Behne *et al.*, 1988; Evenson and Sunde, 1988; Wu *et al.*, 1995). Up to 21 of these proteins have been further characterised by purification and/or cloning (Arthur and Beckett, 1994; Hatfield, 2001). Bioinformatic techniques have also been used to search published nucleotide sequences for SECIS elements to identify potential sequences for selenoproteins (Kryukov *et al.*, 1999; Lescure *et al.*, 1999; Martin-Romero *et al.*, 2001). Some potential selenoproteins identified in this manner have been expressed, although in most cases their enzyme activities or functions have not been identified. On the basis of abundant and non-abundant proteins in the genome, it has been suggested that there may be up to 100 selenoproteins (Burk and Hill, 1993).

Of the mammalian selenoproteins that have been characterised (Table 1.03) four are GPXs, three are iodothyronine deiodinases and three are thioredoxin reductases (TRs). All mammalian selenoproteins with known functions are redox enzymes with an active site that contains SeCys (Gasdaska *et al.*, 1999a).

a) Glutathione peroxidases

The family of glutathione peroxidases includes five distinct selenoproteins. Each of these peroxidases arise from distinct gene products which are homologous with one another but structurally and phytogenetically unrelated to the Se-containing bacterial oxidoreductases and the other mammalian selenoproteins. Distinct biological roles of the individual GPX types are suggested from differences in catalytic efficiency, substrate specificity, tissue distribution, subcellular compartmentalisation, and Se-dependency of biosynthesis.

As is common to most known selenoproteins, Se is an essential requirement for the synthesis of all the GPXs. The elimination of the SeCys residue or carboxymethylation of SeCys by iodoacetate inactivates the GPX (Ursini *et al.*, 1995). One of the common features of the GPX family is a strictly conserved catalytic triad of selenocysteine, glutamine and tryptophan residues (Hatfield, 2001).

A novel form of GPX in the skin (Frank *et al.*, 1997) is upregulated by keratinocyte growth factor and produced by fibroblasts and keratinocytes. The protein is putitatively involved in wound repair; however it is not a selenoenzyme (Munz *et al.*, 1997), and the gene has only slight homology to other GPX genes.

Cytoplasmic glutathione peroxidase (cyGPX)

cyGPX comprises four identical subunits, each with a molecular weight between 19-25 kDa. Each subunit contains a glutathione binding site and an active site comprising of a single

SeCys residue which is located at approximately the fortieth residue from the N-terminal end, the exact location depending on the tissue and species (Sunde, 1994; Zachara, 1992). cyGPX is expressed in virtually all cells although its specific activity is known to vary between different species and tissues. The tissue distribution of cyGPX largely parallels oxidative metabolism, being present at high levels in liver, kidney, lung, red blood cells and placenta. Within the cell, it is present as a soluble enzyme of the cytosol and mitochondrial matrix. The antioxidant function of the GPXs is well documented. CyGPX, as well as the other tetrameric GPXs (plGPX and GIGPX), is able to catalyse the reduction of a variety of hydroperoxides, including H_2O_2, *tert*-butyl hydroperoxide, cumene hydroperoxide and fatty acid hydroperoxides (Flohé, 1989), according to the following reactions.

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG$$

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSG + H_2O$$

Ganther proposed that the first step involves the oxidation of the GPX active site selenol (E-SeH) to selenenic acid (E-SeOH) by the peroxide substrate (Ganther, 1999). This is followed by the reaction of selenenic acid with the first GSH molecule to form the sulfoselenide adduct (E-Se-SG) and water (or alcohol in the case of organic hydroperoxides). The final step involves the cleavage of the sulfoselenide link by a second GSH molecule, releasing GSSG and restoring GPX to its selenol form (Zachara, 1992).

The importance of cyGPX in the physiological regulation of intracellular hydroperoxide concentrations is in doubt as Se deficiency, resulting in a loss of cyGPX activity to less than 1% of control values in the rat liver, resulted in no obvious adverse clinical effects (Arthur *et al.*, 1987; Burk and Hill, 1993). In addition cyGPX knock-out mice displayed no clinical abnormalities under normal physiological conditions and no increased sensitivity to hyperoxia (Ho *et al.*, 1997). Therefore, as losses of cyGPX appear to be well tolerated and because cyGPX appears to contain a large fraction of the total body Se, it has been postulated that cyGPX serves as a Se store which can be mobilised for the synthesis of selenoproteins more critical for survival (Arthur and Beckett, 1994; Burk and Hill, 1993; Sunde, 1990). In the situation of Se deficiency, the loss of cyGPX activity may be compensated for by increased levels of GST isoenzymes, which have non-Se dependent peroxidase activity (Coursin and Cihla, 1996).

Table 1.03 Mammalian selenoproteins and their postulated functions

Selenoprotein	Proposed functions
Glutathione peroxidase (GPX)	
Cytosolic GPX	Intracellular antioxidant, Se store?
Phospholipid hydroperoxide GPX	Intracellular antioxidant, structural role in spermatozoa
Plasma GPX	Plasma antioxidant
Gastrointestinal GPX	Gastrointestinal tract antioxidant
Sperm nuclei GPX	Sperm maturation and male fertility
Iodothyronine deiodinase	
Types I & II	Catalyses the conversion of thyroxine (T4) to active 3,5,3' triiodothyronine (T3)
Types I & III	Catalyses the conversion of T4 to 3,3',5' reverse triiodothyronine
Selenoprotein P	Transport? Antioxidant role?
Selenoprotein W	Antioxidant role?
Thioredoxin reductase (TR)	Multiple roles associated with its role as part of a dithiol-disulphide oxidoreductase system
TR1, TR2/TRß, TR3	
Selenophosphate synthetase 2	Catalyses the production of selenophosphate, required for selenoprotein synthesis
Selenoproteins (R, X), T, N	SelR is methionine sulphoxide reductase (R and X same protein). T and N unknown role
15-kDa selenoprotein	Linked with prostate cancer?
15-kDa selenoprotein	T cells
18-kDa mitochondrial selenoprotein	Unknown?
34-kDa nuclear selenoprotein	Antioxidant?

However, the role of cyGPX as a potential antioxidant against the pathogenic effect of ROS in other conditions has not been ruled out. For example, oxidative stress induced by 30 mg/kg of paraquat, which is approximately LD_{20} for mice, killed all the cyGPX knock-out mice, whereas control mice showed no signs of toxicity at this dose (de Haan *et al.*, 1998). In the same study neurons from cyGPX knock-out mice were more sensitive to toxicity from H_2O_2 (de Haan *et al.*, 1998). In another study cyGPX knock-out mice, mice over-expressing cyGPX, or control mice received an intraperitoneal injection of paraquat (12.5, 50, or 125 mg/kg body weight) (Cheng *et al.*, 1998). Survival time was greatly reduced in cyGPX knock-out mice compared to control mice, but only in the presence of adequate dietary Se (0.3 mg/kg). The mean survival time of mice over-expressing cyGPX was 10-fold longer than control mice. The cyGPX knockout mouse (-/-) is also more susceptible to diquat-induced oxidative stress (Fu *et al.*, 1999), and myocardial ischaemia reperfusion (Yoshida *et al.*, 1997) than WT mice. The GPX overexpressing mouse is more resistant to damage by paraquat or diquat than WT mice (Lei, 2001).

Levels of cyGPX, as well as PHGPX, increase after excisional wounding of skin in mice, suggesting a role for the GPXs in detoxification of ROS during cutaneous wound repair (Steiling *et al.*, 1999).

Phospholipid hydroperoxide glutathione peroxidase (PHGPX)

PHGPX is a monomeric selenoprotein with a molecular mass of approximately 19 kDa (Ursini *et al.*, 1985). cDNA sequencing of PHGPX shows 45 % homology to cyGPX, although the location of the SeCys residue is in a region of high homology to cyGPX (Zachara, 1992). PHGPX is present in the cytosol of different tissues as well as being associated with membranes (Roveri *et al.*, 1994). It exhibits a different distribution pattern to cyGPX with a relatively high abundance in rat testis (Burk and Hill, 1993). PHGPX also appears to account for the majority of GPX activity in the brain (Hatfield, 2001); however activities do not consistently match protein measurements, suggesting the occurrence of enzymatically inactive forms.

PHGPX, like cyGPX, can function as an antioxidant with the ability to reduce fatty acid hydroperoxides as well as cholesterol hydroperoxides (Ursini *et al.*, 1985). As a monomer, the active site of PHGPX is much more accessible. This may help to explain the broad specificity of PHGPX for hydroperoxides comprising H_2O_2, phosphatidyl choline hydroperoxide and cholesterol ester hydroperoxides even when these are integrated into lipoproteins or cell membranes (Ursini *et al.*, 1995). Transfection of PHGPX into cells provides protection from injury mediated by lipid hydroperoxides (Yagi *et al.*, 1996a) and cholesterol hydroperoxides (Arai *et al.*, 1999; Hurst *et al.*, 2001; Imai *et al.*, 1996). It has been estimated by kinetic modelling that PHGPX is far more efficient than cyGPX in inactivating lipid hydroperoxides (Girotti, 2001).

PHGPX plays an important role in spermatogenesis. In spermatids PHGPX exists as a soluble peroxidase exhibiting high activity (Ursini *et al.*, 1999). However during spermatogenesis PHGPX polymerises to become an enzymatically inactive, oxidatively cross-linked insoluble protein which is tightly bound to the mitochondrial capsule (Ursini *et al.*, 1999). This may explain the increased fragility of the mitochondrial mid-piece observed in Se-deficiency.

Protection of membranes from oxidative damage is only one, and possibly not the most important, role of PHGPX. PHGPX may also be an important biological regulator, reducing lipoxygenases, inhibiting apoptosis, and suppressing cytokine-induced NFκB activation (as with the other members of the GPX family). PHGPX may have a role in modulating leukotriene biosynthesis (Huang *et al.*, 1998; Imai *et al.*, 1998; Weitzel and Wendel, 1993).

Plasma glutathione peroxidase (plGPX)

plGPX is an extracellular enzyme, and is distinct enzymatically and structurally from cyGPX. PlGPX is a glycoprotein with subunits of between 21.5 -23 kDa (Zachara, 1992). The main site of synthesis is the proximal tubules of the kidney (Whitin *et al.*, 1998; Yoshimura *et al.*, 1991). PlGPX is found in the plasma, chamber water of the eye, and amniotic fluid (Takahashi *et al.*, 1990). PlGPX has been implicated in the reduction of lipid hydroperoxides in LDL (Saito *et al.*, 1999; Yamamoto and Takahashi, 1993); however the substrate specificity of plGPX is not ideal for reduction of peroxidised LDL since it does not reduce peroxidised cholesterol esters (Yamamoto and Takahashi, 1993).

Gastrointestinal glutathione peroxidase (GI-GPX)

GI-GPX is closely related to cyGPX in terms of its sequence homology, tetrameric structure and substrate specificity (Chu *et al.*, 1993) and is found in the epithelium lining the gastrointestinal tract (Chu *et al.*, 1993; Esworthy *et al.*, 1998). GI-GPX has been proposed to be responsible for the protection of the gastrointestinal tract from the adverse effects of ingested hydroperoxides (Chu *et al.*, 1993; Esworthy *et al.*, 1998), and may be of importance in the prevention of colon cancer (Chu *et al.*, 1997).

Sperm nuclei glutathione peroxidase (snGPX)

This 34kDa selenoprotein is present in spermatozoa and testis (Behne *et al.*, 1988; Behne *et al.*, 1997; Pfeifer *et al.*, 2001). Its sequence at the N-terminus includes a signal for localization within the nuclei, where it is the only selenoenzyme present (Pfeifer *et al.*, 2001). The concentration of snGPX in Se-deficient rats is depleted to one third of control levels, and there is resulting severe disruption of chromation condensation (Behne and Kyriakopoulos, 2001; Pfeifer *et al.*, 2001). snGPX is essential for maturation of sperm and male fertility (Pfeifer *et al.*, 2001).

b) Thioredoxin reductases

The thioredoxin reductases (TRs) belong to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases that includes lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase. However the other members of this family lack the SeCys residue which characterises TR as a selenoprotein (Gladyshev *et al.*, 1996; Tamura and Stadtman, 1996). TR, in conjunction with its substrate thioredoxin (Trx) and NADPH as a cofactor, forms a powerful dithiol-disulphide oxidoreductase system (the 'thioredoxin system') which has multiple functions and is ubiquitous.

Mammalian TR has different characteristics from the enzyme of bacteria, yeast or plants, exhibiting a much broader substrate specificity, and having subunits of 55 kDa instead of 35 kDa (Arnér *et al.*, 1999). The bacterial TR does not have the SeCys-containing redox-active motif of the mammalian enzyme.

Isoforms of Human TR

TR1

The most abundant TR isozyme in mammalian cells is the ubiquitous cytoplasmic form, TR1 (Sun *et al.*, 1999); TR1 was only shown to be a selenoenzyme fairly recently (Tamura *et al.*, 1995; Tamura and Stadtman, 1996). TR1 is located largely in the cytosol and rarely in the nucleus, and is also secreted from granules of endocrine and exocrine cells (Rozell *et al.*, 1985). The highest levels of TR1 are found in testis, ovary, and placenta (Koishi *et al.*, 1997; Miranda-Vizuete *et al.*, 1999b; Sun *et al.*, 1999). Human enzyme preparations have given Mr ranging from 54.6 kDa for purified placental TR1, which lacks Met and Asn residues at the N-terminal (Mustacich and Powis, 2000), to 56 kDa for TR1 isolated from T cells (Gladyshev *et al.*, 1996). Potential variants of this isoenzyme have been described which are due to alternative splicing of the first exons of the TR1 gene (Rundlöf *et al.*, 2000; Sun *et al.*, 2001).

TR2

A second TR isoform, variously referred to as TRβ (Gasdaska *et al.*, 1999b) and TrxR2 (Lee *et al.*, 1999), has also been identified and characterized, and shown to be a mitochondrial enzyme (Lee *et al.*, 1999; Miranda-Vizuete *et al.*, 1999a; Miranda-Vizuete *et al.*, 1999b; Rigobello *et al.*, 1998). This isoform of human TR, differs in molecular mass (56.2 - 56.5 kDa) from TR1 and exhibits a distinct pattern of tissue expression, with high levels of TRβ/TR2 mRNA found in the prostate, testis, liver, uterus and small intestine, and only low levels in placenta, kidney, pancreas, thymus and peripheral blood leukocytes (Gasdaska *et al.*, 1999b; Miranda-Vizuete *et al.*, 1999b). The cDNA sequence of TRβ is identical to that of TR2 from human adrenal except that the former possesses a Met-Ala-Ala extension at its N-

terminus (Gorlatov and Stadtman, 1999). TRβ has been identified in both the cytosolic and microsomal subcellular fractions of MCF-7 human breast cancer cells (Gasdaska *et al.*, 1999b).

TR2 has 84 % similarity at the protein level, and ~ 55 % sequence similarity to TR1 (Gasdaska *et al.*, 1999b; Miranda-Vizuete *et al.*, 1999a). TR2 has a 33 amino acid N-terminal extension, which is recognised as a mitochondrial import sequence. Localisation of TR2 in mitochondria is eliminated upon removal of the N-terminal extension (Miranda-Vizuete *et al.*, 1999b). The TR2 isoform is postulated to provide mitochondria-specific defence against ROS produced by the mitochondrial respiratory chain, thus maintaining a redox balance critical for cell survival (Lee *et al.*, 1999). This theory is furthered by the recent identification of a unique mitochondrial form of Trx (Spyrou *et al.*, 1997) and by the discovery of a mitochondrial thioredoxin peroxidase (Watabe *et al.*, 1997), the induction of Trx in mitochondria by oxidative stress (Gauntt *et al.*, 1994), and also the Trx-mediated regulation of SOD synthesis (Das *et al.*, 1997). Substrates of TR can induce mitochondrial swelling *in vitro* (Wudarczyk *et al.*, 1996). The mitochondrial Trx system could also conceivably act as an electron donor for mitochondrial GPX (Miranda-Vizuete *et al.*, 1999a).

TR3

A third Secys-containing TR, referred to as TR3 (Sun *et al.*, 1999), was purified from [^{75}Se]-labelled mouse testis, where it is preferentially expressed (Sun *et al.*, 1999). The deduced sequence of the human enzyme shows > 50 % identity to that of TR1. It contains a long N-terminal extension, and has a higher molecular mass (~65kDa) than the other two isoenzymes.

Common properties of the isoforms of human TR

All three homodimeric isoforms of TR share considerable sequence homology, including a conserved -Cys-Val-Asn-Val-Gly-Cys redox-active (residues Cys^{59} and Cys^{64}) catalytic site at the N-terminal, FAD-binding and NADPH-binding domains, and a dimer interface domain (Miranda-Vizuete *et al.*, 1999b; Sun *et al.*, 1999). This conserved active-site sequence motif is also found in human glutathione reductase, and is located in the FAD domain of the enzymes, whereas in the TR of *E. coli* the catalytic site, -Cys-Ala-Thr-Cys-, is part of the NADPH domain (Zhong *et al.*, 1998).

The C-terminal end of mammalian TR contains an extension of 16 amino acid residues with a SeCys as the penultimate residue within the sequence -Gly-Cys-SeCys-Gly- (Cys^{497}-SeCys^{498}). This sequence is conserved in all mammalian TR isoforms reported to date (Zhong *et al.*, 1998) (Gasdaska *et al.*, 1999b; Gladyshev *et al.*, 1996; Lee *et al.*, 1999; Miranda-Vizuete *et al.*, 1999b). The SeCys residue is essential for the catalytic activity of TR; its removal by carboxypeptidase digestion (Zhong *et al.*, 1998), trypsin digestion

(Gromer *et al.*, 1998b) or modification by alkylation (Gorlatov and Stadtman, 1998; Zhong *et al.*, 1998) leads to inactivation. A Cys^{497} /Cys^{498} mutant of TR1 and a truncated mutant lacking residues 498 and 499 had decreased or no catalytic activity, respectively, compared with native enzyme (Gasdaska *et al.*, 1999a). The replacement of SeCys with Cys in the rat TR results in about 1 % of the activity seen in the native enzyme with Trx as substrate, and major loss in k_{cat} (Zhong and Holmgren, 2000). Mutant enzymes (SECIS removal, SeCys substitution, and truncated enzyme) also lack hydroperoxidase activity (Zhong and Holmgren, 2000).

Distribution of TR in vivo

The principal site of TR activity is the cytosol, with some localisation of the enzyme in the perimembraneous area of the plasma membrane (Hansson *et al.*, 1986; Rozell *et al.*, 1985) as well as the granular endoplasmic reticulum and cisternae of the Golgi body (Rozell *et al.*, 1988) in the rat cell. The localisation of TR to these areas may be vital for maintaining Trx in its reduced form. All TR isoforms are synthesized in the cytosol (Gromer *et al.*, 1999). The mitochondrial form is then translocated into mitochondria. TR in human liver is detected in nuclei, mitochondria, lysosomes, microsomes, and cytosol (Chen *et al.*, 2002).

Structure

Each monomer of TR includes a tightly-bound FAD group, an NADPH-binding site and an active site containing a redox-active disulphide. The three-dimensional structure of rat TR has been elucidated (Sandalova *et al.*, 2001). The C-terminal extension of amino acid residues containing SeCys is proposed to fold in such a way as to be able to advance toward the disulphide at the active site of the other subunit of the TR dimer. The active sites are situated at the interface between the subunits, thus the dimer is the 'functional unit' of TR.

Mechanism

The enzyme mechanism of reduction of a substrate by TR involves the transfer of electrons from NADPH, via FAD, to the active-site disulphide formed by the cysteine residues in positions 59 and 64 within the sequence -Cys-Ala-Thr-Cys-, which then goes on to reduce the substrate (Arscott *et al.*, 1997; Gromer *et al.*, 1998b; Lee *et al.*, 2000b; Zhong *et al.*, 2000). Overall, the thiol-redox control is a stepwise reduction whereby TR catalyses the transfer of an electron from NADPH to Trx, which then reduces a number of protein thiol acceptors.

The C-terminal end of human TR may be flexible, allowing the -Cys-SeCys-Gly moiety to carry reducing equivalents from the conserved active-site Cys residues to the substrate (Gromer *et al.*, 1999; Gromer *et al.*, 1998b). The extension at the C-terminus of TR may lengthen the electron transport pathway from the catalytic disulphide to the surface of the enzyme, where it can react with Trx (Sandalova *et al.*, 2001).

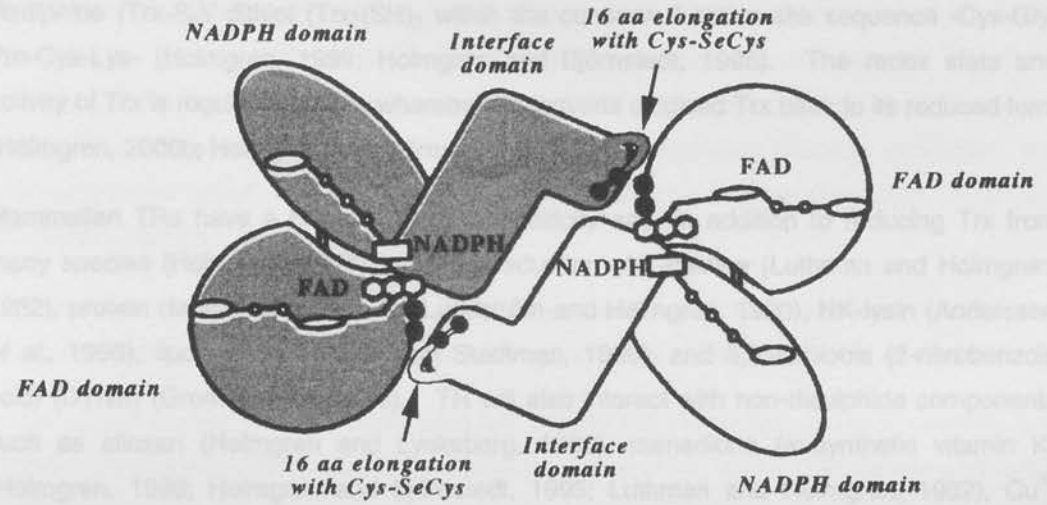


Figure 1.05 Proposed model of mammalian TR (from (Zhong *et al.*, 2000)). The C-terminal elongation of 16 amino acid residues extends from the interface, positioning residues SeCys[498] and Cys[497] in one subunit adjacent to Cys[59] and Cys[64] (redox-active disulphide/dithiol) of the other subunit. Electrons can then be transferred out from the redox-active disulphide/dithiol to the SeCys and Cys residues, which make up the active site of the enzyme. The active sites of TR are located at the interface between the two subunits, and the dimer is therefore the functional unit of the enzyme. The redox-active disulphide is located in the FAD domain.

Substrates

Trx is a ubiquitous low molecular weight (10-12 kDa) multifunctional protein (Holmgren, 1985; Holmgren, 1989; Holmgren and Björnstedt, 1995). It comprises a redox-active disulphide (Trx-S_2)/ dithiol (Trx-$(SH)_2$ within the conserved active site sequence -Cys-Gly-Pro-Cys-Lys- (Holmgren, 1989; Holmgren and Björnstedt, 1995). The redox state and activity of Trx is regulated by TR, whereby TR converts oxidised Trx back to its reduced form (Holmgren, 2000b; Holmgren and Björnstedt, 1995).

Mammalian TRs have a broad substrate specificity and, in addition to reducing Trx from many species (Holmgren, 1977), catalyze reduction of L-cystine (Luthman and Holmgren, 1982), protein disulphide isomerase (Lundström and Holmgren, 1990), NK-lysin (Andersson *et al.*, 1996), lipoic acid (Tamura and Stadtman, 1996), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Gromer *et al.*, 1999). TR will also interact with non-disulphide components such as alloxan (Holmgren and Lyckeborg, 1980), menadione (a synthetic vitamin K) (Holmgren, 1999; Holmgren and Björnstedt, 1995; Luthman and Holmgren, 1982), Cu^{2+} (Holmgren and Lyckeborg, 1980), selenite (Björnstedt *et al.*, 1995b; Björnstedt *et al.*, 1996; Holmgren, 1999), selenodiglutathione (Björnstedt *et al.*, 1995b; Ganther, 1999), methylseleninate (Gromer and Gross, 2002), lipid hydroperoxides (Björnstedt *et al.*, 1995a), hydrogen peroxide (Björnstedt *et al.*, 1995a; Gromer *et al.*, 1999; Zhong and Holmgren, 2000) and the SeCys residue at the active site of plasma glutathione peroxidase (Björnstedt *et al.*, 1994; Holmgren, 1999). S-Nitrosoglutathione (GSNO), an important metabolite and transport form of nitric oxide (NO), is also a substrate of mammalian TR (Becker *et al.*, 2000). The ability of TR to reduce dehydroascorbic acid as well as the ascorbyl radical has also been reported (May *et al.*, 1998a; May *et al.*, 1997). The majority of studies investigating substrates of TR have used TR purified according to the method of Holmgren (Holmgren, 1977). This method uses a primary centrifugation step of 16,000 *x g*, after which the supernatant is saved and further purified. Since mitochondria spin down at 6,500 *x g* in rat liver homogenate (Rice and Lindsay, 1997), it is likely that the majority of the studies quoted have investigated the cytosolic form of TR alone.

The C-terminal catalytic SeCys may provide an explanation for the broad substrate specificity of mammalian TR, allowing the enzyme to reduce bulky proteins as well as small molecules.

Regulation of Expression

The expression of TR1 appears to be regulated through a number of factors including Se supply (Berggren *et al.*, 1997; Berggren *et al.*, 1999; Gallegos *et al.*, 1997; Marcocci *et al.*, 1997; Yarimizu *et al.*, 2000), redox state of the cell (Sun *et al.*, 1999), oxidative stress (Das *et al.*, 1999; Didier *et al.*, 2001; Eftekharpour *et al.*, 2000; Ejima *et al.*, 1999a; Lechner *et al.*,

2002) including $ONOO^-$ (Park *et al.*, 2002), calcium (Gitler *et al.*, 2002), and also through activation of protein kinase C (PKC) (Anema *et al.*, 1999; Kumar and Holmgren, 1999). Increases in Se supply (Berggren *et al.*, 1997; Berggren *et al.*, 1999; Gallegos *et al.*, 1997; Yarimizu *et al.*, 2000) and oxidative stress (Didier *et al.*, 2001; Eftekharpour *et al.*, 2000; Ejima *et al.*, 1999a; Lechner *et al.*, 2002) lead to increased expression of TR, whilst activation of PKC may decrease (Anema *et al.*, 1999) or increase (Kumar and Holmgren, 1999) the expression of the enzyme. The GPX-mimic ebselen promotes the hydroperoxidase activity of TR. Using H_2O_2 as a substrate, ebselen stimulates TR activity (Holmgren, 2000a).

TR1 levels are well maintained when Se supply is low, but protein levels do not increase dramatically under conditions of Se excess (Berggren *et al.*, 1999; Gasdaska *et al.*, 1999a).

In cell culture studies both TR expression and activity are dependent on Se availability, due in part to enhanced mRNA stabilisation by Se (Gallegos *et al.*, 1997). The degree of change in TR activity and TR protein levels is not always directly related (Berggren *et al.*, 1999; Gallegos *et al.*, 1997). In specific rat tissues increases in TR activity did not directly correlate with increases in protein levels; instead they paralleled increases in specific activity, possibly due to an increased SeCys incorporation (Berggren *et al.*, 1999). These findings are consistent with the possibility that in Se-deficiency a truncated protein is formed in which translation is terminated at the UGA codon where SeCys is normally inserted (Gladyshev *et al.*, 1996).

Biological Roles of TR

The reduction of Trx ($Trx\text{-}S_2$) to $Trx\text{-}(SH)_2$ catalysed by TR (reaction *a*) provides a powerful protein disulphide reductase (reaction *b*) which has multiple roles. Interest in the TR/Trx system increased after the initial discovery that TR provides reduced Trx which serves as a hydrogen donor for ribonucleotide reductase in the initial and rate limiting step in DNA synthesis (Thelander and Reichard, 1979).

$$NADPH + H^+ + Trx\text{-}S_2 \xrightarrow{TR} NADP^+ + Trx\text{-}(SH)_2 \qquad (a)$$

$$Trx\text{-}(SH)_2 + protein\text{-}S_2 \xrightarrow{spontaneous} Trx\text{-}S_2 + protein\text{-}(SH)_2 \qquad (b)$$

The TR/ Trx system has subsequently been associated with a multitude of disparate cellular functions including the regulation of cell growth in both normal and cancer cells (possibly by increasing the sensitivity of the cell to endogenous growth factors) (Berggren *et al.*, 1996; Freemerman *et al.*, 1999; Gallegos *et al.*, 1996; Oblong *et al.*, 1994) and the inhibition of

apoptosis (regulation of apoptosis signalling kinase-1) (Fujiwara *et al.*, 1999). The TR/ Trx system is thought to regulate gene expression through activation of DNA-binding activity of transcription factors (Berggren *et al.*, 1996; Gallegos *et al.*, 1997; Holmgren, 2000b; McKenzie *et al.*, 2002a), including NF-κB (Matthews *et al.*, 1992) and the glucocorticoid receptor (Makino *et al.*, 1996), and the modulation of activator protein 1 (AP-1) activity (Karimpour *et al.*, 2002) indirectly through a nuclear redox factor ref-1/HAPE (Hirota *et al.*, 1997). The TR/Trx system can regulate the cellular redox state (Holmgren, 2000b) (for example, TR can serve as a redox regulator of cellular antioxidant systems by acting as an electron donor for methionine sulphoxide reductase (Holmgren, 1985), peroxiredoxin (Chae *et al.*, 1999), protein disulphide isomerase (Holmgren and Björnstedt, 1995), and vitamin K epoxide reductase (Silverman and Nandi, 1988), and protect against oxidative stress (Holmgren, 1985; Holmgren, 1989; Holmgren, 2000a; Holmgren and Björnstedt, 1995), including chemotherapeutic agents (see below). The TR/Trx system also facilitates the refolding of disulphide-containing proteins (Lundström and Holmgren, 1990) and modifies the structure of export proteins. Oxidation of the redox-active SeCys residue of TR contributes to redox signalling and to mediating responses to oxidative stress (Sun *et al.*, 1999).

Detoxification and Antioxidant Functions of TR

TR can reduce and detoxify lipid hydroperoxides, H_2O_2, and organic hydroperoxides directly using NADPH as a cofactor in a cell-free system (Björnstedt *et al.*, 1995a). The accumulation of these compounds in tissues exerts deleterious effects, e.g. the hydroperoxide (15S)-hydroperoxy-(5Z), (8Z), 11(Z), 13(E)-eicosatetranoic acid ((15S)-HPETE) oxidises LDL to a cytotoxic form, with implications in atherogenesis (Ylä-Herttuala *et al.*, 1990). Detoxification of (15S)-HPETE and other LOOH is through a GPX-mediated reduction. However, the TR pathway operating together with catalytic amounts of SeCys has a high capacity to detoxify such compounds and may provide an important alternative to the Se-dependent GPXs for detoxification (Björnstedt *et al.*, 1995a). To serve as an efficient peroxidase in the cell, continued regeneration of the selenol (-SeH) form of the SeCys residue in TR from the oxidised –SeOH form is necessary to permit enzyme turnover (Gorlatov and Stadtman, 1999). The rate of peroxide reduction *in vitro*, with H_2O_2 as substrate, has been calculated to be much lower than that of an effective peroxidase such as cyGPX (Gorlatov and Stadtman, 1999). However, studies by Zhong *et al.* (Zhong and Holmgren, 2000) have calculated a high apparent K_m of TR for H_2O_2 (2.5 mM), which would suggest a role for TR only when there is an elevated H_2O_2 level. TR reduces H_2O_2 and 15-HPETE at approximately the same rate (Björnstedt *et al.*, 1995a).

TR can replace GSH as an electron donor to regenerate the active site SeCys residue in GPXs, which is oxidised during the peroxidase reaction (Björnstedt *et al.*, 1994). TR can regenerate bioactivity in proteins inactivated by oxidative stress, such as glyceraldehdye-3-

phosphate dehydrogenase (Fernando *et al.*, 1992), phosphotyrosine phosphatase (McCarty, 1999) and nitric oxide synthase (Ejima *et al.*, 1999b; Patel *et al.*, 1996). TR can also function as a peroxynitrite reductase (Arteel *et al.*, 1999a), and efficiently reduce ebselen selenoxide back to ebselen. NK-lysin is a pore-forming peptide secreted by natural killer cells, which is efficiently reduced and inactivated by human TR, confining the effects of the highly toxic substances to invading microorganisms (Andersson *et al.*, 1996). TR in conjunction with Trx is important in antioxidant protection of the human placenta (Ejima *et al.*, 1999b), and protects mouse placentae from oxidative damage (Ejima *et al.*, 1999a). TR is reported to be highly expressed on the surface of human keratinocytes and melanocytes, where it is suggested to provide the first line of defence in the skin against ROS generated in response to UV light (Schallreuter and Wood, 1986) (see section 1.11.1 for details of TR in the skin).

The TR system as well as Trx or TR alone can protect against oxidative stress. Up-regulation of the thioredoxin system has been associated with the development of cellular resistance to the chemotherapeutic agent *cis*- diamminedichloroplatinum (II) (Sasada *et al.*, 2000; Sasada *et al.*, 1999). Human Trx protects murine endothelial cells from injury induced by H_2O_2 (Nakamura *et al.*, 1994) and reperfusion injury (Isowa *et al.*, 2000). Human hepatoma cells with increased Trx show decreased sensitivity to cell kill by cisplatin (Kawahara *et al.*, 1996), and adult-T cell leukemia cell lines with higher Trx levels show lower resistance to doxorubicin (Wang *et al.*, 1997). Recombinant human Trx protects against H_2O_2- and tumour necrosis factor-alpha (TNFα)-induced cytoxicity (Matsuda *et al.*, 1991; Nakamura *et al.*, 1994). Trx can prevent oxidative stress-induced apoptosis in cells (Andoh *et al.*, 2002). Trx is also a specific electron donor for many peroxiredoxins (Berggren *et al.*, 2001; Chae *et al.*, 1999), which are crucial for the reduction of peroxides. Trx protects the lens from oxidative stress and cataract formation (Reddy *et al.*, 1999), helps protect the intestinal epithelium from oxidative stress (Higashikubo *et al.*, 1999), may protect the developing fertilized egg and placental trophoblasts from oxidative damage in the uterus (Kobayashi *et al.*, 1995), and prevents reperfusion-induced arrhythmias in an isolated rat heart model (Aota *et al.*, 1996). In *in vivo* animal models of ischaemia reperfusion, Trx can protect the lung (Yagi *et al.*, 1994; Yokomise *et al.*, 1994) and brain (Takagi *et al.*, 1999) from damage. Extracellularly, increased levels of Trx in plasma have been found in a collection of diseases including hepatocellular carcinoma, pancreatic ductal carcinoma, AIDS, Sjögrens Syndrome, and rheumatoid arthritis (Powis and Montford, 2001).

In baboon lung, Trx and TR are expressed constitutively at low levels in the foetus, and increase rapidly with the onset of O_2 or air breathing at birth in a possible protective role (Das *et al.*, 1999). Increases in TR and Trx mRNA are also observed in adult baboon lung explants exposed to 95 % O_2.

Interaction with other antioxidant systems

TR also has effects on other antioxidant systems, e.g. TR in the presence of Trx leads to a specific induction of Mn-SOD (Das *et al.*, 1997). The Trx system can reduce a variety of protein disulphide groups and other substrates important in maintaining the redox balance in cells. The Trx/TR system is capable of directly regenerating ascorbate from dehydroascorbate (DHA) (May *et al.*, 1997) and the ascorbyl free radical (May *et al.*, 1998a). TR can reduce the ascorbyl free radical to ascorbate with a K_m of 2.8 μM, which is in the physiological range for this free radical in cells sustaining oxidative stress (Buettner and Jurkiewicz, 1993; May *et al.*, 1998a). Since the α-tocopherol radical is reduced non-enzymatically by ascorbate (Liebler, 1993), coupling of these reactions provides a theoretical mechanism for the *in-vivo* regeneration of α-tocopherol from the oxidized form via TR and protein disulphide isomerase (Tamura *et al.*, 1995). Se and vitamin E have mutual sparing effects, and TR could be the link explaining this phenomenon (Tamura *et al.*, 1995). Se can spare ascorbate and α-tocopherol in liver cell lines exposed to oxidative stress (Li *et al.*, 2001). Trx also contributes to the induction of HO-1 by inflammatory mediators (Wiesel *et al.*, 2000). Production of excessive amounts of NO in the cell is counteracted by its conjugation with GSH, resulting in formation of GSNO. GSNO can subsequently be directly cleaved by TR or by the TR/Trx system, releasing GSH and NO (Nikitovic and Holmgren, 1996). TR may also reduce the antioxidants lipoic acid (Arnér *et al.*, 1996; Watabe *et al.*, 1999) and ubiquinone (Xia *et al.*, 2001).

TR inhibitor studies and implications for TR in pathophysiology

TR activity is inhibited by gold compounds (Gromer *et al.*, 1998a; Hill *et al.*, 1997), which are used to treat some autoimmune diseases such as rheumatoid arthritis (Van Riel *et al.*, 1986). Similarly, the anti-tumour drugs quinones, doxorubicin and diaziquone also inhibit TR activity (Mau and Powis, 1992). Treatment with gold drugs increases oxidative stress (Reglinski *et al.*, 1997). Rheumatoid arthritis, a common chronic disease characterized by persistent inflammation in the synovial membranes of peripheral joints (Kerimova *et al.*, 2000), may involve secretion of ROS and cytokines (e.g. tumour necrosis factor-α). The synovial fluid from inflamed joints of patients with rheumatoid arthritis have significantly increased levels of Trx compared with levels in patients with other rheumatic diseases (osteoarthritis, gout and reactive arthritis) (Maurice *et al.*, 1999). *In-situ* mRNA hybridisation confirmed the presence of TR in rheumatoid synovium also. Trx levels were found to be higher in erosive joints than in non-erosive joints. Stimulation of synovial synoviocytes with H_2O_2 led to an induction of increased Trx, suggesting a role for local oxidative stress in upregulated Trx production in the inflamed joints of patients of rheumatoid arthritis. However, contrasting results of down-regulation of antioxidant enzymes in rheumatoid arthritis are reported (Kerimova *et al.*,

2000), and the pathogenic mechanisms are far from clear. Severe rheumatoid arthritis has also been associated with Se deficiency (Tarp *et al.*, 1985).

c) Iodothyronine deiodinases (IDI)

The iodothyronine deiodinases (IDI) are a family of three selenoproteins, all of which possess a single SeCys residue at the active site, and are involved in the metabolism of thyroid hormones by removal of iodine from iodothyronine substrates (St Germain and Galton, 1997). The Trx/TR system can reduce the IDIs to an active form (Bhat *et al.*, 1989; Sharifi and St Germain, 1992). The deiodinases are thought to be important in regulating foetal development as well as regulating thyroid hormone metabolism in the adult.

d) Selenoprotein P (SelP)

Selenoprotein P (SelP) is an extracellular glycoprotein, and is the major plasma selenoprotein, accounting for most of the Se in human plasma (Mostert *et al.*, 1998). SelP contains up to ten SeCys residues per 43 kDa polypeptide chain, nine of which are situated at the carboxyl terminus (Burk and Hill, 1994; Hill and Burk, 1994). It has been proposed to have a Se transport function (Motsenbocker and Tappel, 1982), and new data from a selP knockout mouse confirms this (Hill *et al.*, 2002).

Selenoprotein P is also associated with endothelial cells in the liver, where it may provide the endothelium with important defence against oxidative damage (Burk *et al.*, 1997).

e) Selenoprotein W

Selenoprotein W is a small intracellular selenoprotein containing one SeCys residue per polypeptide chain (Burk and Hill, 1999), and four different isoforms of selenoprotein W have been isolated from rat muscle, with molecular masses between 9.5 -10 kDa (Allan *et al.*, 1999). The brain, muscle, testis and spleen contain the greatest amounts of selenoprotein W (Burk and Hill, 1999). The catalytic activity of selenoprotein W is at present unknown although an antioxidant role has been postulated (Burk and Hill, 1999). A possible antioxidant function is further strengthened by the discovery that glutathione is bound to the major selenoprotein W species (Beilstein *et al.*, 1996; Gu *et al.*, 1999), and that overexpression of selenoprotein W in cultured cell lines reduces their sensitivity to H_2O_2 cytotoxicity (Jeong *et al.*, 2002).

f) Selenophosphate synthetase

Sps2 is a selenoprotein (Guimaraes *et al.*, 1996). The requirement of *Sps2* synthesis for Se would suggest that this protein is involved in the regulation of selenoprotein synthesis *per se*, such that Se-deficiency would be reflected by a down-regulation of *Sps2* expression subsequently with a parallel decrease in the expression of other selenoproteins, possibly providing auto-regulation of selenoprotein synthesis.

g) Other selenoproteins and Se-containing proteins, and novel proteins

Selenoproteins

Investigation using SDS-PAGE and two-dimensional electrophoresis of [^{75}Se]-labelled animal tissue has identified approximately 35 Se-containing proteins or protein subunits with molecular masses ranging between 6 and 116 kDa, five of which contain Se as a SeCys residue (Behne *et al.*, 1988; Behne and Kyriakopoulos, 2001; Behne *et al.*, 1999). Some of these proteins may represent as yet uncharacterized SeCys-containing selenoproteins. The selenoproteins S, T, (R or X), Z and a 15 kDa protein from prostate and a more ubiquitous 15 kDa protein, a 34 kDa nuclear protein and a further 18 kDa mitochondrial protein have all been either purified or identified by bioinformatic methods (Hatfield, 2001; Kyriakopoulos *et al.*, 2000; Lescure *et al.*, 1999). Their functions have yet to be described in totality, but their existence further emphasises the wide range of metabolic processes that can potentially be influenced by changes in Se status. The 15 kDa selenoprotein that has been isolated and characterized in human T cells, with high expression in prostate tissue (Gladyshev *et al.*, 1998b), has been linked with the protective role of Se against prostate cancer (Gladyshev *et al.*, 1998b; Kumaraswamy *et al.*, 2000). Selenoprotein R has been identified as a zinc-containing methionine sulphoxide reductase (Kryukov *et al.*, 2002).

Se-containing proteins

Several Se-containing proteins have been characterized in which Se is bound covalently in a form which is not SeCys. The biological significance of the Se in these proteins is unknown and the activity of these proteins is not regulated by Se status. Fatty acid-binding proteins (FABP) are involved in the regulation of intracellular levels of fatty acids (Masouyé *et al.*, 1997). A 14 kDa mouse liver Se-binding protein identified as an FABP (Bansal *et al.*, 1989) is proposed to be involved in regulation of cell growth. Protein disulphide isomerase (PDI), a Se-containing protein, is involved in protein folding. Se is tightly bound to PDI, but is not incorporated as a SeCys residue (Sinha *et al.*, 1993). The role of Se in this protein is unclear since PDI activity is not regulated by Se in either cultured cells or rats (Arthur *et al.*, 1991; Sinha *et al.*, 1993).

1.2.8 Regulation of selenoprotein expression

Hierarchy of selenium supply

Se supply regulates selenoprotein translation, mediated by Sec-tRNA availability. Se deficiency results in a decrease in all selenoproteins, unlike other modulators of selenoprotein expression which may be specific for certain selenoproteins. The extent to which Se availability effects selenoprotein expression differs between tissues and between individual selenoproteins within a tissue (Behne *et al.*, 1988; Bermano *et al.*, 1996a; Bermano *et al.*, 1995; Burk and Hill, 1993; Hill *et al.*, 1997; Weitzel *et al.*, 1990).

There is a distinct hierarchy in the Se supply to different tissues during Se deficiency. Regulatory mechanisms exist to ensure that in Se deficiency, Se levels are maintained in certain priority organs. Se is generally retained by organs such as the brain, endocrine and reproductive organs, indicating the relative importance of the element for the biological functions of these organs, whilst it is rapidly lost from liver, kidneys and muscle (Behne *et al.*, 1988; Bermano *et al.*, 1995). This differential regulation is not achieved by a decrease in the turnover of Se in deficient tissues alone; re-distribution of the metabolised element and priority supply to these tissues are also involved.

In addition there is an important hierarchy of Se supply to different selenoenzymes within tissues in Se deficiency (Behne *et al.*, 1988; Bermano *et al.*, 1996a; Burk and Hill, 1993; Hill *et al.*, 1997; Weitzel *et al.*, 1990), such that Se supply to selenoproteins other than GPX has priority (i.e. loss of cyGPX expression occurs before most other selenoproteins) (Bermano *et al.*, 1995).

All selenoproteins are regulated by Se at the level of translation. Se status does not affect the transcription rate of genes for any selenoproteins characterised (Burk and Hill, 1993; Wingler *et al.*, 1999). However, changes in the expression and/or the activity of selenoproteins in Se deficiency are accompanied by changes in mRNA levels which may result from alterations in mRNA translation and/ or stability (Bermano *et al.*, 1996a; Bermano *et al.*, 1995; Gallegos *et al.*, 1997; Saedi *et al.*, 1988; Sunde *et al.*, 1993). CyGPX expression is uniquely regulated significantly by Se at the levels of translation and mRNA stability; the regulation of mRNA stability involves nonsense-mediated mRNA decay (Hatfield, 2001). The hierarchy of Se regulation of selenoprotein mRNA quantity is (most to least regulation): cyGPX >> IDI-1 > TR1 > SelP, PHGPX (Hatfield, 2001). The hierarchy of Se regulation of selenoprotein translation is (most to least regulation): cyGPX > IDI-1 > SelP, TR1 > PHGPX. The overall hierarchy of the GPXs is: GI-GPX > PHGPX > plGPX > cyGPX (Brigelius-Flohé, 1999).

In addition, Se deficiency may also result in premature polypeptide chain termination due to recognition of the UGA codon as a normal stop codon (Burk and Hill, 1993). Se-deficiency

can lead to a decrease in the levels of mRNA for TR and to incorporation of cysteine instead of SeCys into the protein, resulting in a less active form of the protein (Gallegos *et al.*, 1997). Se depletion in some cell types leads to a decreased half-life of cyGPX mRNA but not PHGPX mRNA (Bermano *et al.*, 1995; Bermano *et al.*, 1996b). Sequences in the 3′ UTRs of mRNA allow regulation of expression by a variety of mechanisms, including alterations in mRNA turnover, translation initiation, subcellular localization and by controlling the choice between SeCys insertion or termination of protein synthesis (Berry *et al.*, 1994).

Other modulators of selenoprotein expression

Factors other than Se status modulate individual selenoprotein expression. For example, the expression of TR is increased by PMA, the calcium ionophore A23187 and ROS(Howie *et al.*, 1998; Kumar and Holmgren, 1999; Sun *et al.*, 1999). Iodine-deficiency in adult rats increases cyGPX activity in the thyroid (Mitchell *et al.*, 1996). The cyGPX activity is also increased in vascular endothelial cells by fatty acids and interleukin-1β (Crosby *et al.*, 1996). p53 expression results in elevated cyGPX expression, but down-regulation of TR expression (Gladyshev *et al.*, 1998a). p53-induced activation of transcription of GPX has been shown in other studies (Tan *et al.*, 1999), as has the down-regulation of TR (Polyak *et al.*, 1997). These studies suggest that regulation of selenoprotein expression can be p53-dependent, and that TR and cyGPX can be regulated independently. Expression of selenoproteins *in vivo* is also subject to transcriptional control by hormones such as gonadotropin (Brigelius-Flohé *et al.*, 1994).

1.3 ATHEROSCLEROSIS

1.3.1 Introduction

Atherosclerosis (Gr. "porridge-like hardening") is a highly complex vascular disease which leads to myocardial and cerebral infarction, gangrene and loss of function of the extremities (Ross, 1993b). It is the principal cause of morbidity and mortality in the United States, Europe, and much of Asia (Ross, 1999). The mechanisms involved in the development of the atheromatous lesion have been established.

1.3.2 'Response to injury' hypothesis

The most widely accepted model of atherogenesis is the 'response-to-injury' hypothesis formulated by Ross and Glomset (Ross, 1993b; Ross, 1993a; Ross and Glomset, 1973). Briefly, the theory proposes that injury to the endothelium (for example, by local disturbances of blood flow at certain branch points of the arterial tree) coupled with major risk factors (such as hypercholesterolemia, hyperglycemia, cigarette smoking, and microbial infections) can initiate a protective, inflammatory-fibroproliferative response. This response results in a series of compensatory cellular and molecular events leading to the formation and development of the atherosclerotic lesion. Multiple interactions of monocytes, T-lymphocytes and platelets together with the endothelium and smooth muscle of the arterial wall are involved in the process.

1.3.3 The 'Oxidative-Modification' Hypothesis

This hypothesis suggests that in addition to endothelial activation, oxidative modification of low density lipoprotein (LDL) plays a pivotal role in atherogenesis (Esterbauer *et al.*, 1992; Heinecke, 1998; Steinberg *et al.*, 1989). Hessler *et al.* observed that LDL could injure cells in culture (Hessler *et al.*, 1979), and that the injury depended upon oxidative modification of LDL (Hessler *et al.*, 1983; Morel *et al.*, 1984; Morel *et al.*, 1983). The oxidative modification hypothesis of atherosclerosis (Steinberg *et al.*, 1989) originated with the observation by Goldstein *et al.* (Goldstein *et al.*, 1979) that cultured macrophages are converted to lipid-laden foam cells in the presence of chemically-modified LDL, but not native LDL. Subsequent work revealed that all the major cell types of the arterial wall (endothelial cells, smooth muscle cells, and macrophages) oxidatively modify LDL (Chisolm and Steinberg, 2000) to a form that is recognised by scavenger receptors on macrophages (Henriksen *et al.*, 1981; Henriksen *et al.*, 1983).

The LDL oxidative modification hypothesis itemizes on one hand the lipid peroxidation theory, and incorporates the need for the inflammatory cell-cell interactions proposed in Ross' 'response-to-injury' hypothesis.

1.3.4 The lesions of atherosclerosis

The lesions of atherosclerosis are principally found in large and medium-sized elastic and muscular arteries such as the femoral artery, cerebral arteries, aorta and coronary arteries. The more classical nomenclature of atherosclerotic lesions, the fatty streak, intermediate lesion and fibrous plaque (Ross, 1995), has been superseded by 'Stary's classification' (Yutani *et al.*, 1999). The 'Stary classification' embraces the more recent morphological and biochemical details of atherogenesis.

Stary's classification

Stary's classification characterises atherosclerotic lesions by categorising their progression into five phases, each being defined by its histological characteristics (table 1.04) (Stary *et al.*, 1995). The initial type I lesion consists of macrophage-derived foam cells containing lipid droplets. The location of these and subsequent lesions is more evident in locations such as branches, bifurcations and curvatures, and regions of arterial narrowing which are subject to alterations in blood flow (Ross, 1999). These 'adaptive thickenings' do not cause substantial morphological alteration. Type II lesions (formerly 'fatty streaks') consist of layers of macrophage foam cells and SMC with intracellular lipid droplets. In the type III lesion pools of extracellular lipids also occur, which serve as the precursor to the core of lipid that typifies type IV lesions. The lipid core may now also contain thick layers of fibrous connective tissue and/or lipid or calcium (type V lesion). The morbidity and mortality associated with atherosclerosis are predominantly due to the rupture of types IV and V lesions, with ensuing hematoma and thrombus formation occluding the vessel lumen.

1.3.5 Cellular interactions of atherosclerosis

One of the earliest events in atherogenesis is a change in the endothelial surface phenotype. The expression of molecules on the endothelium responsible for the adherence, accumulation and migration of monocytes and T-cells markedly increases at specific locations within the artery wall. Adhesion molecules expressed, including L- E- and P-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Gimbrone, 1995; Ross, 1999), allow adherence of monocytes and T-lymphocytes to the endothelium. An increase in permeability in the endothelium, mediated by factors such as oxidised low density lipoprotein (oxLDL), monocyte chemotactic protein-1 (MCP-1), and platelet derived growth factor (PDGF), aids the migration of these inflammatory cells into the sub-endothelial intima. Expression of growth-regulatory molecules (e.g. PDGF, fibroblast growth factor-2, transforming growth factor-β), and cytokines (e.g. TNFα and interleukin-2), which may activate EC and SMC of the lesion, can be modified by the presence of macrophages and T lymphocytes in the intima. Ultimately the release of various growth factors and cytokines from cells in the lesion leads to progression of the fatty streak into a more advanced, complicated lesion involving formation of a fibrous plaque.

Table 1.04 Stary's classification of atherosclerotic lesions. The flow diagram indicates the sequence of progression of human atherosclerotic lesions. The Roman numerals represent the different classification of lesion as defined in the left hand column and the direction of the arrows indicate the sequence in which characteristic morphologies may change. Cycling between stages V and VI may represent the natural progression or growth of the atherosclerotic lesion. Adapted from Stary *et al.* (Stary *et al.*, 1995).

nomenclature & main histology	sequence in progression	main growth mechanism
type I (initial lesion) isolated macrophage foam cells	I	growth mainly by lipid accumulation
type II (fatty streak lesion) mainly intracellular lipid accumulation	II	
type III (intermediate) lesion type II changes & small extracellular lipid pools	III	
type IV (atheroma) lesion type II changes & core of extracellular lipid	IV	
type V (fibroatheroma) lesion lipid core & fibrotic layer, or multiple lipid cores & fibrotic layers, or mainly calcific, or mainly fibrotic	V	accelerated smooth muscle and collagen increase
type VI (complicated) lesion fissure or rupture hematoma-hemorrhage, thrombus	VI	thrombosis, hematoma

1.4 THE ENDOTHELIUM

1.4.1 Structure and function

Arterial vessels exhibit a layered structure of tissues and cells (figure 1.06). The outermost layer (tunica adventitia) comprises connective tissue rich in elastic and collagen fibres, whilst the middle layer (tunica media) consists of connective tissue and SMC. Adjacent to the lumen is the tunica intima consisting of lamina propria and a basement membrane upon which lies the endothelium.

The vascular endothelium is a continuous monolayer of cells that lines the inner surface of all blood vessels, providing a barrier between blood and the underlying SMC of the vessel. The endothelium is a highly dynamic, metabolically active structure with numerous endocrine, paracrine and autocrine functions. Owing to its positioning at the interface between the blood and tunica intima the endothelium has a vital role in the maintenance of vascular homeostasis, with functions ranging from the regulation of the mass transport of solutes or macromolecules across vessel walls to immune responses and control of vascular tone. The principal functions of the endothelium are listed in table 1.05.

1.4.2 Endothelium-derived factors

The functions of the endothelium are mainly mediated directly or indirectly by endothelium-derived autacoids such as $NO^{\bullet}$, prostacyclin (PGI_2) (a member of the prostaglandin family), an endothelium-derived hyperpolarizing factor (EDHF), thromboxane A_2 (TXA_2), endothelin-1 (ET-1), $O_2^{\bullet-}$, and possibly prostaglandin H_2. These factors modulate the functions of SMC and platelets as well as the EC from which they were derived. Endothelium-derived factors are secreted in response to autonomic and sensory nerves (e.g. acetylcholine, noradrenalin, substance P), circulating hormones (e.g. catecholamines, angiotensin II), products of coagulation (e.g. serotonin, adenosine diphosphate, thrombin), autocoids produced by EC or SMC (e.g. bradykinin, angiotensins, ET-1) and to changes in shear stress exerted on the endothelium by the circulation.

ROS are released in response to shear stress and some agonists (e.g. bradykinin) (Mombouli and Vanhoutte, 1999), and can inactivate $NO^{\bullet}$, favouring vasoconstriction. ROS may also mediate vasoconstriction by facilitating the mobilisation of Ca^{2+} in vascular SMC and/or by enhancement of Ca^{2+} sensitivity of contractile elements.

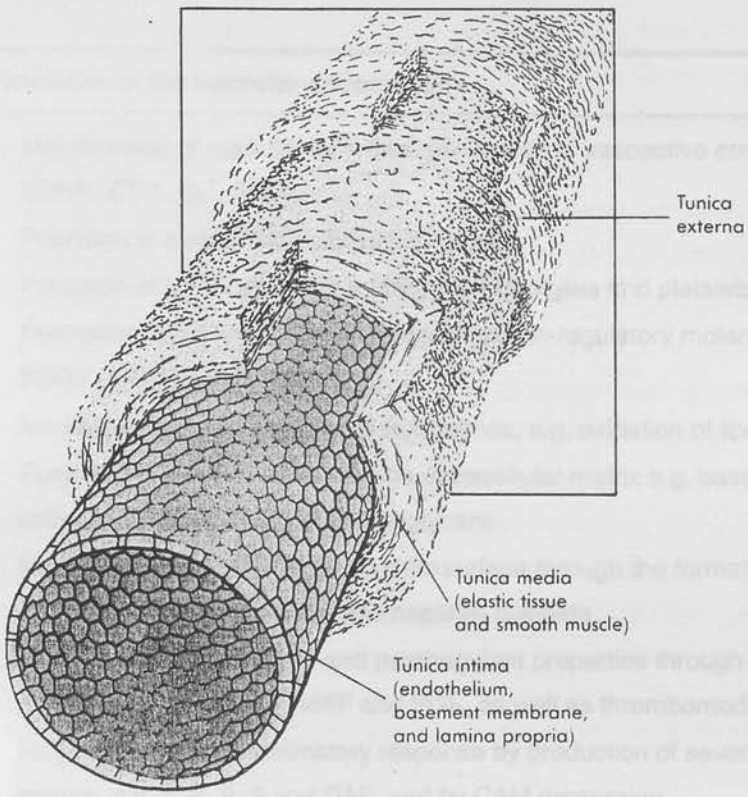


Figure 1.06 Structure of arterial vessel wall. From Carola *et al.* (Carola *et al.*, 1992). The outermost layer (tunica externa or adventitia) consists of connective tissue abundant in elastic and collagen fibres, whilst the middle layer (tunica media) consists of connective tissue and smooth muscle cells. Adjacent to the lumen is the tunica intima consisting of lamina propria and a basement membrane upon which lies the endothelium.

Table 1.05 Functions of the vascular endothelium

Functions of the vascular endothelium
• Maintenance of vascular tone through release of vasoactive compounds, e.g. $NO^{\bullet}$, PGI_2, EDHF, ET-1, $O_2^{\bullet-}$, PGH_2
• Provision of a selective permeability barrier
• Provision of a non-adherent surface for leukocytes and platelets
• Formation and secretion of a variety of growth-regulatory molecules and cytokines, e.g. PDGF, bFGF and TNFα
• Modification of plasma-derived compounds, e.g. oxidation of lipoprotein
• Formation and maintenance of the extracellular matrix e.g. basement membrane, collagen, elastic fibres and proteoglycans
• Maintenance of a non-thrombogenic surface through the formation of molecules such as $NO^{\bullet}$, PGI_2, thrombomodulin and heparan sulphate
• Provision of anticoagulant and procoagulant properties through the production of molecules such as $NO^{\bullet}$, vWF and PGI_2, as well as thrombomodulin and t-PA/u-PA
• Regulation of the inflammatory response by production of several proinflammatory agents, e.g. IL-6, IL-8 and PAF, and by CAM expression

Abbreviations: BFGF, basic fibroblast growth factor; CAM, cell adhesion molecule; EDHF, endothelium-derived hyperpolarizing factor; ET-1, endothelin-1; IL, interleukin; NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; PAF, platelet activating factor; PDGF, platelet-derived growth factor; PGH_2, prostaglandin H_2; PGI_2, prostacyclin; t-PA, tissue type plasminogen activator; TNFα, tissue necrosis factor alpha; u-PA, urokinase type plasminogen activator, vWF; von Willebrand factor.

1.5 ENDOTHELIAL DYSFUNCTION IN ATHEROSCLEROSIS

1.5.1 Introduction

There is an abundance of evidence demonstrating that although the endothelium in atherosclerotic vessels from animals and humans is physically intact, there are a number of apparent functional modifications. In healthy arteries, the endothelium generally performs an inhibitory role, inhibiting SMC contraction, monocyte adhesion, oxidation of LDL, platelet adherence and aggregation, synthesis of inflammatory cytokines, vascular smooth muscle proliferation and thrombosis (Shimokawa, 1999). However the endothelium also plays an important role in the pathophysiology of several vascular diseases including atherosclerosis. Due to its anatomical position the endothelial surface is a primary target for attack from damaging stimuli from the earliest stages of atherogenesis. Endothelial dysfunction, i.e. 'the shut-down or inactivation of certain intrinsic anti-atherogenic mechanisms' (Busse and Fleming, 1996), is a primary step in atherogenesis. (Ross and Glomset, 1973) and is observed prior to the development of overt atheroma, suggesting a principal role for endothelial dysfunction in the initiation of atherogenesis. Endothelial dysfunction is detectable in patients with a family history of atherosclerosis prior to the manifestation of atherogenesis (Busse and Fleming, 1996).

A number of risk factors, alone or in combination, can bring about 'endothelial dysfunction'. Risk factors which promote atherogenesis (e.g. elevated and modified LDL; cigarette smoking; ageing; gender; menopause; physical inactivity; hypertension; ischemia; diabetes mellitus; elevated plasma homocysteine levels and infectious organisms such as herpes virus or *Chlamydia pneumoniae)* (Ross, 1999; Shimokawa, 1999) are strongly associated with endothelial dysfunction in that the modification or removal of the risk factor (e.g., cholesterol lowering, cessation of smoking, exercise) improves endothelial dysfunction and may prevent further disease progression (Vogel, 1997; Vogel, 1999).

1.5.2 Features and mechanisms of endothelial dysfunction

The endothelial dysfunction that results from injury caused by one or more risk factors alters the normal homeostatic properties of the endothelium (table 1.05), thus promoting atherogenesis (Vogel, 1997). There is considerable evidence to suggest that endothelial dysfunction is associated with the loss of $NO^{\bullet}$ production and/or its bioavailability (Busse and Fleming, 1996; Harrison, 1997a). Recent evidence suggests that endothelial dysfunction is characterised not simply by a loss of $NO^{\bullet}$ production by endothelial nitric oxide synthase (eNOS), but by dysregulation of this enzyme, which may involve perturbations in subcellular trafficking, phosphorylation, cofactor availability, and/or interactions with other proteins. $NO^{\bullet}$ plays a crucial role in the maintenance of vascular homeostasis under normal conditions such that the loss of production and/or bioavailability of $NO^{\bullet}$ from the endothelium should lessen the protective effects and predispose the vessel to atherogenesis.

Tetrahydropbiopterin (BH_4), an essential cofactor for all NOS enzymes, is thought to determine whether electron flow within the enzyme can be directed to L-arginine. In intact blood vessels depleted of BH_4, eNOS transfers electrons to the now preferred substrate molecular oxygen to produce $O_2^{\bullet-}$, (Cosentino and Katusic, 1995; Kinoshita *et al.*, 1997). The addition of BH_4 to recombinant eNOS increases $NO^{\bullet}$ production whilst decreasing $O_2^{\bullet-}$ production (Wever *et al.*, 1998). Depletion of BH_4 is associated with vascular pathology (Cosentino *et al.*, 1998). In hypercholesterolemic patients supplementation with BH_4 can improve endothelium-dependent vasodilation (Shimokawa, 1999; Wever *et al.*, 1998). Oxidation of BH_4, by H_2O_2, is reversible by TR (Schallreuter and Wood, 2001) (see section 1.11).

The reduction in $NO^{\bullet}$ bioavailability is proposed to be linked with its increased inactivation. The interaction between $NO^{\bullet}$ and $O_2^{\bullet-}$ effectively reduces the biological activity of $NO^{\bullet}$ due to formation of $ONOO^-$ (figure 1.07). The presence of $ONOO^-$-modified protein in human atherosclerotic lesions and early subintimal streaks has been demonstrated (Beckmann *et al.*, 1994). A two- to three-fold increase in the release of total nitrogen oxides from atherosclerosed rabbit aortas compared to normal controls has been detected, despite a decrease in the bioavailability of $NO^{\bullet}$, implying that the $NO^{\bullet}$ has sustained oxidative destruction (Minor *et al.*, 1990). The reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ to form $ONOO^-$ (figure 1.08) (Beckmann *et al.*, 1994; Harrison, 1997a; Harrison, 1997b) is approximately three times faster than that of scavenging of $O_2^{\bullet-}$ by SOD (Harrison, 1997b). This suggests that under oxidative stress the functions of $NO^{\bullet}$ are inhibited by its conversion to $ONOO^-$, promoting atherogenesis. The Cu/Zn-SOD plays an important role in protecting $NO^{\bullet}$ in the endothelium (Harrison, 1997b; Lynch *et al.*, 1997), maintaining endothelial function. In a rat model of SOD deficiency, a 58 % increase in vascular $O_2^{\bullet-}$ production compared to control rats, and 10-fold less sensitivity of thoracic aorta to relaxation by acetylcholine was reported (Lynch *et al.*, 1997). Pharmacologic inhibition of SOD results in release of $NO^{\bullet}$ from the endothelium in an oxidatively inactive form (Harrison, 1997b).

In cultured EC exposure to hypoxia and high concentrations of oxLDL decrease eNOS expression (Busse and Fleming, 1996; Harrison, 1997a). Oxidised LDL destabilises the mRNA encoding eNOS, decreasing its expression (Liao *et al.*, 1995).

In summary, the findings from animal and human studies suggest that the inactivation of $NO^{\bullet}$ by $O_2^{\bullet-}$ and other ROS observed under pathophysiological conditions contributes to endothelial dysfunction (Busse and Fleming, 1996; Harrison, 1997a). The involvement of ROS in atherogenesis is discussed in further detail in section 1.5.3.

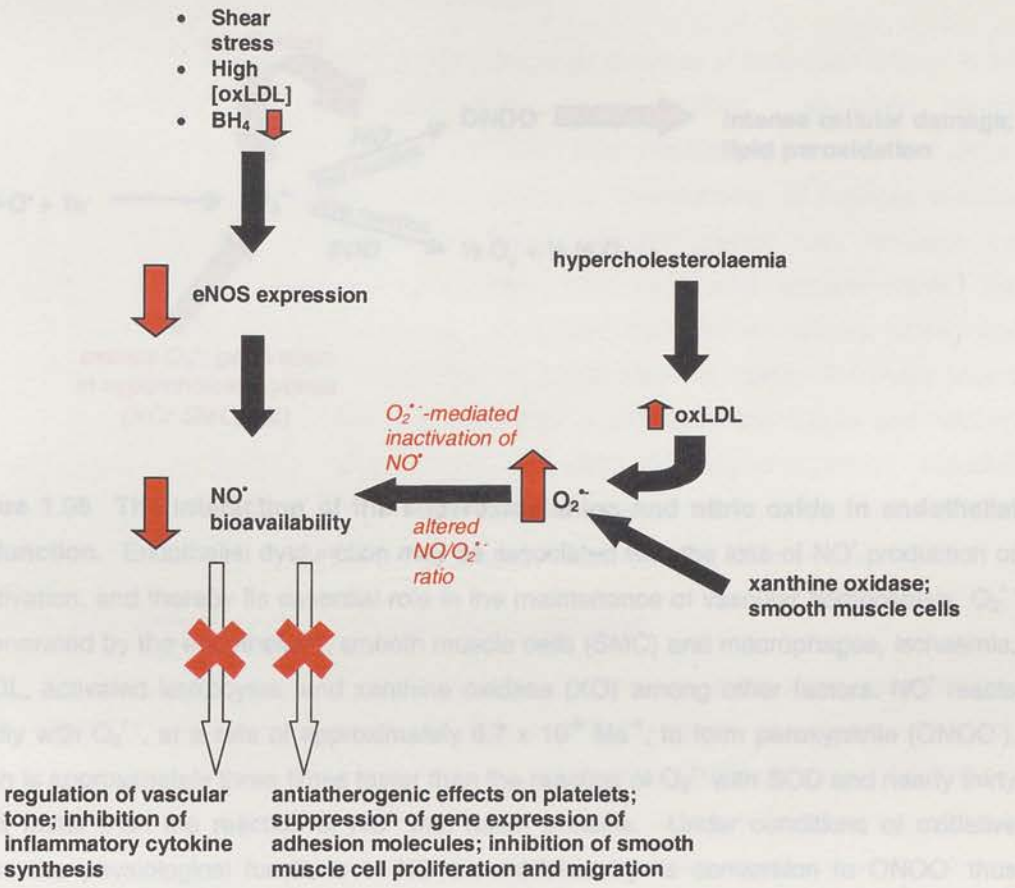


Figure 1.07 Schematic diagram of some of the interacting factors involved in the decreased bioavailability of nitric oxide in the process of endothelial dysfunction. ENOS expression may be decreased by oxidised LDL (oxLDL), hypoxia, shear stress or decreased tetrahydropbiopterin (BH_4) levels. Increases in $O_2^{\bullet -}$ mediate decreased bioavailability of $NO^{\bullet}$, by oxidative destruction and conversion to $ONOO^{-}$. $O_2^{\bullet -}$ is generated by the endothelium, SMC and intimal macrophages in response to injury, ischemia, oxLDL and activated leukocytes. Both $NO^{\bullet}$ and $O_2^{\bullet -}$ are produced constitutively, so changes in the relative flux of either species will modulate NO-dependent endothelial function.

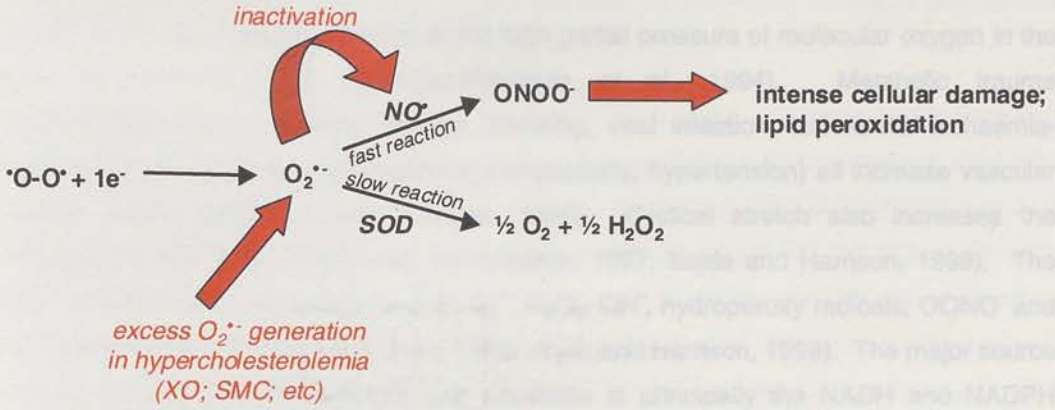


Figure 1.08 The interaction of the superoxide anion and nitric oxide in endothelial dysfunction. Endothelial dysfunction may be associated with the loss of $NO^{\bullet}$ production or inactivation, and thereby its essential role in the maintenance of vascular homeostasis. $O_2^{\bullet -}$ is generated by the endothelium, smooth muscle cells (SMC) and macrophages, ischaemia, oxLDL, activated leukocytes, and xanthine oxidase (XO) among other factors. $NO^{\bullet}$ reacts rapidly with $O_2^{\bullet -}$, at a rate of approximately 6.7×10^{-9} Ms^{-1}, to form peroxynitrite ($ONOO^-$), which is approximately three times faster than the reaction of $O_2^{\bullet -}$ with SOD and nearly thirty times faster than the reaction of $NO^{\bullet}$ with haem proteins. Under conditions of oxidative stress the physiological functions of NO are inhibited by its conversion to $ONOO^-$ thus promoting atherogenesis. $O_2^{\bullet -}$ has several pro-atherogenic features including the oxidation of LDL and lipid accumulation in the vessel wall. $O_2^{\bullet -}$ can also provide a source of other ROS (H_2O_2, OH^-, singlet oxygen, hypochlorous acid and $ONOO^-$) which may participate in lipid peroxidation and influence endothelial function. The altered ratio of $NO/O_2^{\bullet -}$ production has been proposed to diminish the intrinsic inhibition of the transcription factor NFκB, leading to an enhanced expression of endothelial adhesion molecules and chemotactic factors.

1.5.3 Reactive oxygen species and the endothelium

a) Reactive oxygen species and the endothelium

The EC *in vivo* is constantly exposed to the high partial pressure of molecular oxygen in the blood as well as ROS (Schuppe-Koistinen *et al.*, 1994). Metabolic trauma (hypercholesterolemia, diabetes mellitus, smoking, viral infection, ischaemia/ ischaemia-reperfusion) and physical trauma (coronary angioplasty, hypertension) all increase vascular oxidative stress (McGorisk and Treasure, 1996). Cyclical stretch also increases the production of $O_2^{\bullet-}$ by EC (Hishikawa and Lüscher, 1997; Kojda and Harrison, 1999). The ROS relevant to vascular biology include $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$, hydroperoxy radicals, $OONO^-$ and lipid hydroperoxides (Hennig and Chow, 1988; Kojda and Harrison, 1999). The major source of ROS in the normal endothelium and adventitia is principally the NADH and NADPH oxidases (Kojda and Harrison, 1999; Somers *et al.*, 2000; Sorescu *et al.*, 2001). Potential sources of vascular $O_2^{\bullet-}$ are more numerous in pathological conditions, and xanthine oxidase is implicated as a significant source of $O_2^{\bullet-}$ in hypercholesterolemia (Ohara *et al.*, 1993).

b) Reactive oxygen species and endothelial dysfunction

Damage to the endothelium by ROS, free radicals and oxidised lipids and lipoproteins has been shown to favour atherogenesis (Hennig and Chow, 1988; Hennig *et al.*, 2001). Oxidative stress can disrupt several important physiological functions of the endothelium, including regulation of blood flow, inhibition of leukocyte adhesion and platelet aggregation. Oxidative stress may also alter the expression of other genes associated with atherogenesis such as eNOS, ICAM-1, E-selectin, PDGF and FGF-2. The increased presence of $O_2^{\bullet-}$ and loss of $NO^{\bullet}$ bioavailability in hypercholesterolemia results in the activation of genes controlling mechanisms such as monocyte adhesion (Maxwell *et al.*, 1998; Ohara *et al.*, 1993).

Both atherosclerosis and hypercholesterolemia are associated with increased flux of $O_2^{\bullet-}$ within the vascular wall. Excess generation of $O_2^{\bullet-}$ within vessels from hypercholesterolemic subjects may lead to endothelial dysfunction (Mügge *et al.*, 1991; Ohara *et al.*, 1993). In hypercholesterolemic rabbits a three-fold increase in aortic $O_2^{\bullet-}$ production was measured compared to normal rabbits (Maxwell *et al.*, 1998; Ohara *et al.*, 1993). The increased steady-state flux of $O_2^{\bullet-}$ was abolished by endothelial denudation and inhibited by oxypurinol (a non-competitive inhibitor of xanthine oxidase) inferring that the endothelium provides the major source of abnormal $O_2^{\bullet-}$ production in early atherosclerosis through the activation of xanthine oxidase. Hypercholesterolemic patients treated with oxypurinol demonstrate a partial improvement in $NO^{\bullet}$-mediated arterial vasodilation (Cardillo *et al.*, 1997). In hypercholesterolemic rabbits, dietary treatment normalizes vascular $O_2^{\bullet-}$ production, and the bioactivity of endothelium-derived $NO^{\bullet}$ (Ohara *et al.*, 1995). Hypercholesterolemia may also

be associated with increased circulating levels of xanthine oxidase that may bind to the vascular endothelium (White *et al.*, 1996). In the later stages of atherosclerosis activated macrophages in the intima and SMC are also likely to contribute to the production of vascular $O_2^{\bullet-}$ as well as other ROS (Kojda and Harrison, 1999).

Lipid radicals and $O_2^{\bullet-}$ are detected in suspensions of cultured EC treated with chemical oxidative stressors (Rosen and Freeman, 1984). The generation of lipid radicals markedly decreases upon addition of SOD into the system, suggesting that $O_2^{\bullet-}$ radicals make a significant contribution to the production of lipid free radicals, and to EC membrane damage (Rosen and Freeman, 1984). The $O_2^{\bullet-}$ anion has several pro-atherogenic features including the oxidation of LDL and lipid accumulation in the vessel wall. $O_2^{\bullet-}$ can also provide a source of other ROS (H_2O_2, $OH^{\bullet}$, singlet oxygen, HOCl and $ONOO^-$) which may participate in lipid peroxidation and impact upon endothelial function. At neutral pH the peroxynitrite radical is protonated and can form cytotoxic peroxynitrous acid which can spontaneously yield the hydroxyl radical and nitrite (NO_2^-) (Harrison and Ohara, 1995; Wever *et al.*, 1998). The oxidation of LDL, protein fragmentation by nitration of proteins, DNA damage, modification of iron-sulphur clusters, zinc-fingers, protein thiols and membrane lipids are all effects of $ONOO^-$, and may be important in the pathophysiology of atherosclerosis (Graham *et al.*, 1993; Harrison and Ohara, 1995; Heinecke, 1998; Wever *et al.*, 1998).

1.5.4 Oxidised LDL - a promoter of atherosclerosis

Introduction

LDLs are roughly spherical particles with an average diameter of 19-25 nm, and a relative molecular mass of between 1.8 and 2.8 million (Esterbauer *et al.*, 1992). In humans, the hydrophobic core of each particle comprises triglyceride and cholesterol ester, and the surface monolayer consists of phospholipid molecules and a single copy of apoB-100 embedded in the outer layer (Esterbauer *et al.*, 1992; Halliwell and Gutteridge, 1999) (Figure 1.09). In addition, the particles contain unesterified cholesterol. The principal oxidizable lipid, cholesteryl linoleate, is situated in the lipoprotein core (Denicola *et al.*, 2002). LDL also contains several lipophilic antioxidants such as α-tocopherol, and minute amounts of γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Esterbauer *et al.*, 1992). Several plant-derived antioxidant pigments such as lycopene and lutein are also contained in LDL (Halliwell and Gutteridge, 1999).

LDL particles are in a dynamic state, their structure and function being dependent on their lipid composition as well as the conformation of apoB-100. LDL particles are defined within the density limits of 1.019 – 1.063 g/ml. Thus, LDL forms a heterogeneous group of particles varying widely in size, composition and structure. Variability in the chemical and structural composition of LDL may in part mediate the differences in susceptibility between LDL

samples to oxidation (Esterbauer *et al.*, 1992). The LDL composition of a given individual will depend to a considerable extent on diet. LDL particles are the main carriers of cholesterol in the human circulation and are thus key players in cholesterol transfer and metabolism (Olson, 1998). LDLs bind to receptors on the surface of cholesterol-requiring cells, and are internalized, releasing cholesterol within the cells.

Oxidation of LDL

Oxidation of LDL is believed to be crucial to initiation of atherogenesis (Heinecke, 1998; Steinberg *et al.*, 1989; Witzum and Steinberg, 1991). LDL stays in the circulation for several days, during which time it may enter the vascular wall. While in plasma, LDL is relatively protected against oxidation by antioxidants in the plasma such as vitamins C and E, β-carotene, and also by antioxidants contained in the LDL particles themselves. However, the levels of antioxidants in the extracellular space are much lower, so once LDL enters the intima, its phospholipids and fatty acids are susceptible to oxidation. The cells of the artery wall secrete various oxidative products that can seed the LDL trapped in the sub-endothelial space and initiate oxidation (Parthasarathy *et al.*, 1999; Witzum and Steinberg, 1991). Oxidative modification of LDL has been demonstrated *in vivo*, and LDL extracted from the intima includes oxLDL, and antibodies to oxLDL also occur (Hörkkö *et al.*, 2000; Rosenfeld, 1991; Steinberg, 1991; Ylä-Herttuala *et al.*, 1989). Such autoantibodies are found in plaques, complexed with oxLDL (Ylä-Herttuala *et al.*, 1994). There are increases in both oxLDL and circulating antibodies to oxLDL in atherosclerotic patients (Esterbauer *et al.*, 1992; McGorisk and Treasure, 1996; Ylä-Herttuala *et al.*, 1989).

Oxidation of LDL enhances its atherogenicity (Hörkkö *et al.*, 2000) whilst inhibition of such oxidation by antioxidant therapy decreases the progression of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits (Ylä-Herttuala *et al.*, 1990). Antioxidants also reduce the autoantibody titre to modified LDL in hypercholesterolemic rabbits (Schwenke and Behr, 2001). Antioxidants and atherosclerosis are further detailed in section 1.5.6.

In-vivo, it is likely that there are many mechanisms by which LDL is oxidised within the artery wall (Heinecke, 1997). Under conditions of increased oxidative stress, enhanced interaction of $O_2^{\bullet-}$ and $NO^{\bullet}$ may lead to peroxidation of lipids, including LDL via $ONOO^-$ as an intermediate (Graham *et al.*, 1993). *In vitro* studies suggest the formation of oxLDL is mediated in part by the enhanced production of ROS by the cells of the arterial wall.

Lipoxygenases (12/15 lipoxygenase), cyclooxygenases and peroxidases (including myeloperoxidase) are postulated to act as enzymatic catalysts for generation of bioactive lipid-oxidation products at sites of inflammation and vascular disease (Benz *et al.*, 1995; Cathcart and Folcik, 2000; Gaut and Heinecke, 2001; Hazen and Heinecke, 1997; Leeuwenburgh *et al.*, 1997; Podrez *et al.*, 2000).

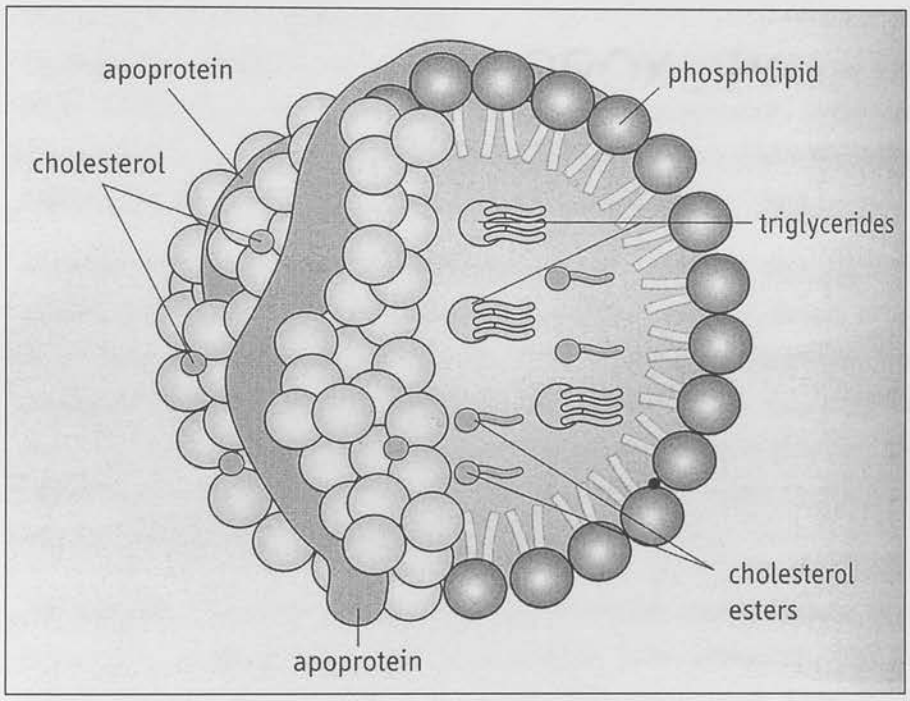


Figure 1.09 The lipoprotein particle. From (Baynes and Dominiczak, 1999). The surface monolayer of the lipoprotein particle comprises unesterified (free) cholesterol (~10%), phospholipids (~20%), and a single copy of apoproteinB-100. Cholesterol esters (~40%), unesterified cholesterol, and triglycerides (~5%) are located in the core of the particle. The quoted percentages are of the whole LDL particle. Protein comprises ~20% of the particle.

The mechanisms by which EC oxidise LDL remain unresolved, with some reports attributing a role to $O_2^{\bullet-}$ alone (Steinbrecher, 1988), lipoxygenase alone (Derian and Lewis, 1992; Parthasarathy *et al.*, 1989), or neither $O_2^{\bullet-}$ nor lipoxyganse (van Hinsberg *et al.*, 1986). Arterial SMC oxidise LDL by releasing $O_2^{\bullet-}$ (Heinecke *et al.*, 1986), while activated human monocytes oxidise LDL by way of a dual superoxide and lipoxygenase-mediated pathway (Cathcart *et al.*, 1989).

The role of oxLDL in atherogenesis

Oxidised LDL affects numerous cellular functions, each of which could contribute to each stage of atherosclerosis. The more established pro-atherogenic properties of oxLDL are summarised in table 1.06. In addition to oxLDL, mmLDL may also elicit biological responses implicated in the pathogenesis of atherosclerosis (Drake *et al.*, 1991).

At higher concentrations oxLDL is directly cytotoxic to several different cell types including endothelial, fibroblasts and SMC in culture (Hessler *et al.*, 1979; Kosugi *et al.*, 1987; Kuzuya *et al.*, 1991; Thomas *et al.*, 1993). This effect is possibly mediated by polar sterols (e.g. oxysterols) generated in the process of LDL oxidation. The mechanism of vascular cell death by oxLDL appears to include both necrosis, secondary to induction of peroxidation of cellular lipids by cholesterol hydroperoxides (Coffey *et al.*, 1995; Thomas *et al.*, 1993), and apoptosis induced by other oxysterols (Lizard *et al.*, 1999).

The principal toxic lipids of oxLDL are present in human lesions (Breuer *et al.*, 1996; Brown *et al.*, 1997; Carpenter *et al.*, 1993; Chisolm *et al.*, 1994) and oxidised forms of lipoproteins from *in vivo* sources are cytotoxic to cultured cells (Hodis *et al.*, 1994; Morel and Chisolm, 1989). Exogenous oxLDL, or its toxic lipids, induce endothelial dysfunction and damage *in vivo* (Harrison and Ohara, 1995; Rangaswamy *et al.*, 1997; Rong *et al.*, 1998).

Biological properties of oxLDL (some of which contribute to the pro-atherogenic properties of this molecule) are summarised in table 1.06.

Table 1.06 Summary of the biological properties of oxLDL. These properties are characteristic of LDL oxidised *in vitro* either by Cu^{++} or endothelial cells for 24 hr. Adapted from (Esterbauer *et al.*, 1992) and (Parthasarathy *et al.*, 1999). Not all the biological properties of oxLDL have been suggested to be atherogenic. Those properties proposed to be pro-atherogenic are marked (*).

Biological properties
• Recognition by macrophage scavenger receptor, resulting in increased uptake and degradation by macrophages; may lead to foam cell formation
• Recognition by unknown proteins (e.g. LOX-1) on cell surface; may account for signalling pathways activated by components of oxLDL
• Possible ceroid (lipid-protein complexes) accumulation in degenerated tissues including macrophage foam cells
• Cytotoxic to several cell types e.g. endothelial, fibroblasts, macrophages and smooth muscle cells (by both necrotic and apoptotic pathways)*
• Chemotactic for monocytes/macrophages, T cells and smooth muscle cells*
• Inhibition of monocyte-macrophage motility and endothelial cell migration*
• Inhibition of NO activation of guanylate cyclase
• Inhibition of relaxation of isolated smooth muscle strips induced by ACh, NO*
• Alteration of the vasoactive compounds released by the endothelium e.g. ET-1*
• Highly antigenic and able to elicit generation of autoantibodies*
• Inhibition of protein C (increases thromboresistance) activity in cultured endothelial cells*
• Suppression of PDGF secretion by monocyte-macrophages
• Increase in macrophage glutathione concentration (approximately two-fold)
• Induction of endothelial-leukocyte adhesion molecules (e.g. VCAM-1, ICAM-1, P-selectin)*
• Induction of chemotactic MCP-1 and colony-stimulating factors
• Induction of DNA synthesis and enhancement of the proliferative response to M-CSF and GM-CSF by macrophages
• Enhancement of pro-coagulant pathways, e.g. by induction of tissue factor and platelet aggregation*
• Stimulation of nuclear transcription factor (NFκB) to increase monocyte adherence*
• Induction of interleukin-1 expression by foam cell macrophages
• Induction of pro-inflammatory genes
• Increase expression of macrophage scavenger receptors, enhancing its own uptake*

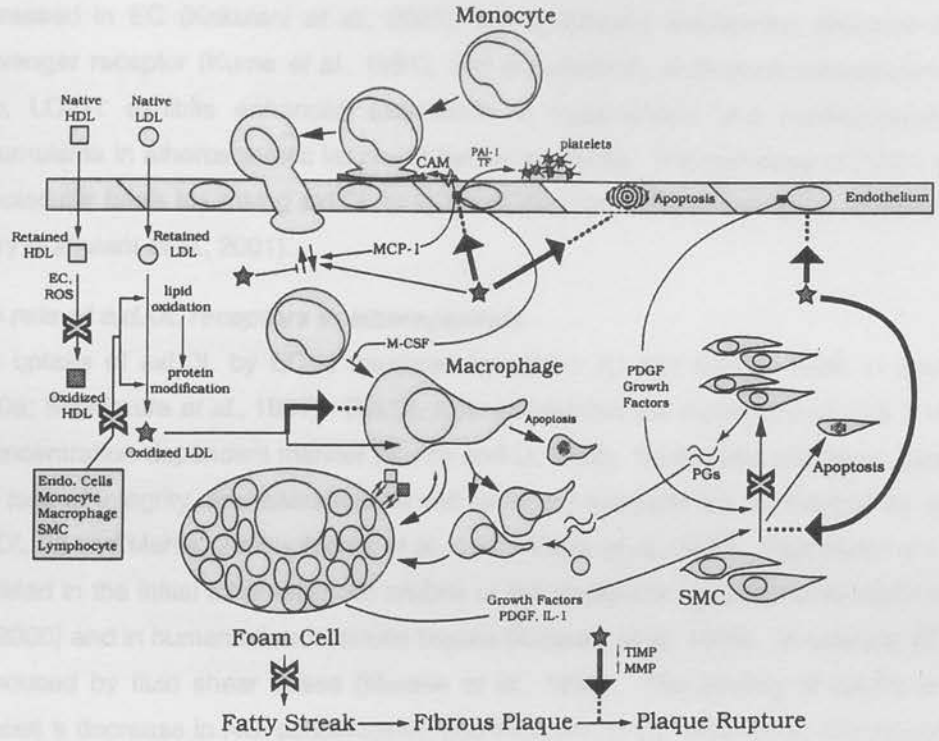


Figure 1.10 Schematic representation of the role that oxidation of LDL might play in the development of atherosclerosis (from (Schwenke, 1998)). Thick arrows and dotted lines indicate direct effects of oxidised LDL. Unfilled crosses indicate processes that are inhibited by antioxidants. Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; CAM, cell adhesion molecule; MCP-1, monocyte chemotactic protein-1; M-CSF, monocyte colony stimulating factor; PDGF, platelet-derived growth factor; IL-1, interleukin-1; PGs, proteoglycans; SMC, smooth muscle cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; EC, endothelial cell; ROS, reactive oxygen species.

Receptors for oxLDL on the endothelial cell

Vascular EC internalise and degrade oxLDL through a unique receptor-mediated pathway, independent of the classic macrophage scavenger receptor (Kume *et al.*, 1991). A novel cell-surface receptor for LDL, the lectin-like oxidised LDL receptor (LOX-1), is predominantly expressed in EC (Kakutani *et al.*, 2001), has a different biochemical structure from the scavenger receptor (Kume *et al.*, 1991), and is putitatively involved in atherosclerosis. *In-vivo*, LOX-1 exhibits enhanced expression in hypertension and hyperlipidaemia, and accumulates in atherosclerotic lesions (Chen *et al.*, 2000). The presence of LOX-1 provides a molecular basis for linking oxLDL to EC, and resultant cellular activation, dysfunction and injury (Kakutani *et al.*, 2001).

The role of oxLDL receptors in atherogenesis

The uptake of oxLDL by EC is mediated by LOX-1 (Li and Mehta, 1999; Li and Mehta, 2000a; Sawamura *et al.*, 1997). OxLDL also upregulates the expression of LOX-1 mRNA in a concentration-dependent manner (Mehta and Li, 1998). Endothelial activation, dysfunction and loss of integrity, and alterations in cell secretory functions are all induced by uptake of oxLDL (Li and Mehta, 2000b; Mehta *et al.*, 1995; Vieira *et al.*, 2000). Expression of LOX-1 is elevated in the initial atherosclerotic lesions of the Watanabe hyperlipidemic rabbit (Chen *et al.*, 2000) and in human atherosclerotic tissues (Kataoka *et al.*, 1999). In vascular EC LOX-1 is induced by fluid shear stress (Murase *et al.*, 1998). The binding of oxLDL to LOX-1 induces a decrease in $NO^{\bullet}$ concentration and induction of $O_2^{\bullet-}$ production (Cominacini *et al.*, 2001), induction of ROS production resulting in activation of NF-kß (Cominacini *et al.*, 2000), the expression of monocyte chemoattractant proteins, and LOX-1 itself (Aoyama *et al.*, 1999; Li and Mehta, 2000b).

Antioxidants and oxidation of LDL

Antioxidants that inhibit oxidation of LDL *in vitro* may have the potential to inhibit atherogenesis and disease progression *in vivo*. A great number of chemicals inhibit the oxidation of LDL *in vitro*, but the efficacy of such compounds has, in most cases, not been tested *in vivo* (Parthasarathy *et al.*, 1999). Inhibition of endothelial cell-mediated oxidation of LDL *in vitro* is further described in chapter three. Antioxidants and atherosclerosis are discussed in section 1.5.6.

1.5.5 Enzymatic antioxidant defence systems in the endothelium

The EC possesses several intracellular enzymatic antioxidant systems, including the glutathione redox system, catalase and SOD (figure 1.02) (Hennig and Chow, 1988). More recently HO and the Trx/TR system have also been implicated in roles of antioxidant defence of the EC (Pohlman and Harlan, 2000).

Whilst both GPX and catalase reduce H_2O_2, catalase has a much lower intracellular concentration in most cells, with the exception of hepatocytes and erythrocytes, and the Km for H_2O_2 is higher than that of GPX (Asahi *et al.*, 1995). Impairment of the glutathione redox cycle, but not catalase, by pharmacologic manipulation increases the susceptibility of pulmonary artery EC to damage by H_2O_2 (Suttorp *et al.*, 1986).

1.5.6 Antioxidants and atherosclerosis

An important implication of the oxidative modification hypothesis of atherosclerosis is that antioxidants may inhibit atherogenesis. The proposed mechanisms for such anti-atherogenic effects are an LDL-specific antioxidant action, i.e. the protection of LDL against oxidative modification, or a tissue- or cell-specific antioxidant action, i.e. increased antioxidant uptake by vascular cells, leading to an elevated antioxidant status (Diaz *et al.*, 1997). The antioxidants may additionally enhance the resistance of vascular cells to the damaging effects mediated by oxLDL.

The most convincing evidence to support the oxidative modification hypothesis of atherosclerosis is the direct demonstration that treatment with a variety of antioxidants (e.g. β-carotene, α-tocopherol, vitamin C, probucol) of hypercholesterolemic animal models results in suppression of atherogenesis (lesion formation) (Diaz *et al.*, 1997; Meagher and Rader, 2001; Schwenke and Behr, 2001).

Basic science and epidemiology are currently strongly supportive of a protective role for vitamin E. Despite the promising results of epidemiological studies, data from the limited number of large prospective clinical trials of antioxidants, in particular vitamin E, in secondary prevention of coronary artery disease have been mixed. The GISSI (1999) and HOPE (2000) trials found no benefit of vitamin E supplementation on cardiovascular disease risk, whilst the SPACE trial (2000) found a 46 % reduction ($p = 0.005$) in the composite primary outcome measure that was primarily attributable to 70 % reduction in acute myocardial infarction in subjects taking vitamin E. The CHAOS study (1996) reported 77% reduction ($p = 0.014$) in non-fatal myocardial infarction in subjects taking vitamin E as compared with those taking placebo (Diaz *et al.*, 1997; Gaziano, 1999; Meagher and Rader, 2001; Tribble, 2001; Witzum and Steinberg, 2001).

Observational studies can only show associations and not causal relationships. Clinical trials also have a number of limitations, including the fact that antioxidant treatment of patients with advanced disease (secondary prevention) may not provide information relevant to disease prevention in healthy individuals (primary prevention). For example, both the GISSI and HOPE trials were secondary prevention trials in which > 75 % of all participants were treated with aspirin or other antiplatelet agents, and many received β-blockers, lipid-lowering drugs, and calcium channel blockers also (Pryor, 2000b). Other possibilities to explain the discrepancies include differences in patient characteristics, degree of depletion of endogenous antioxidant defence mechanisms, the dietary antioxidant content, and potential pro-oxidant effects of vitamins E and C (Meagher and Rader, 2001). It has not yet been conclusively proven that vitamin E acts as an antioxidant *in vivo* (Gaut and Heinecke, 2001; Thomas and Stocker, 2000).

The difficulties in reproducible effects between trials may also be attributed to several causes, among which are: the trials begin too late, involve cure rather than prevention, last too short a time period, and generally involve a single dose of a single antioxidant (Pryor, 2000a), whereas antioxidants act in a synergistic fashion (Pryor, 2000b).

In summary, descriptive, case-controlled and prospective cohort studies have found inverse associations between the frequency of coronary artery disease and dietary intake of antioxidant vitamins. Randomized therapeutic trials have thus far shown no benefit with β-carotene and a possible benefit with vitamin E.

1.6 SE AND CARDIOVASCULAR DISEASE

1.6.1 Se deficiency and cardiovascular disease

The contribution of Se deficiency to the pathogenesis of cardiovascular disease was originally suggested from epidemiological studies that correlated low Se content of forage crops, drinking water and blood levels with regional mortality rates from cardiovascular disease (Schamberger *et al.*, 1979). Such associations are difficult to interpret since it is not possible to exclude the effects of other factors such as interactions of other nutrients, smoking, exercise, alcohol and fat intake, and genetics. Whilst epidemiological studies have provided some evidence for the role of low Se intake in the aetiology of cardiovascular heart disease and ischaemic heart disease, the results of studies within populations have often produced conflicting results. Atherosclerosis is an inflammatory condition, which will provoke the acute-phase response to a degree related to its severity. Being an acute-phase reactant, (Nichol *et al.*, 1998) some decrease in plasma Se concentration may be expected in atherosclerosis patients, even before the occurrence of an event.

An inverse correlation between low plasma Se and the severity of atherosclerosis was found in 91 subjects examined by coronary angioplasty (Moore *et al.*, 1984). In a similar study the ratio of Se to PUFA levels in serum was negatively correlated to the degree of atherosclerosis (Kok *et al.*, 1991). However, Aro *et al.* found no such correlation in a group of Finnish subjects (Aro *et al.*, 1986). The results from eight prospective studies had been published by 1997 which attempted to correlate Se status with risk of coronary vascular disease and myocardial infarction (table 1.07). The results were mixed findings. From the prospective studies of the 1980s, only one found an inverse correlation between Se and the risk of death from cardiovascular death and myocardial infarction, two were equivocal, and no association was demonstrated in the other studies (Levander, 1987; Rayman, 2000). In the 1990s, the results from the Copenhagen Male Study (1992) found that, after adjustments to account for age, cholesterol, social class and smoking, individuals whose Se level was below 1 μmol/L had an increased risk of coronary heart disease (Suadicani *et al.*, 1992). The study carried out by Simonetta *et al.* concluded that there was no association between serum Se and risk of myocardial infarction (Simonetta *et al.*, 1995).

To date, the prospective epidemiological studies linking Se deficiency to cardiovascular disease are inconclusive. Huttunen has postulated that the conflicting data from these studies can be explained by "the threshold effect" of Se intake on the risk of cardiovascular disease (Huttunen, 1997). That is, only populations with a low Se status (serum Se < 45 μg/L), are at risk of cardiovascular disease (Korpela, 1993). The "threshold" is approximately half of that required to maximally express serum GPX. A study of the association between toenail Se and the risk of myocardial infarction in ten centres across nine European countries (Kardinaal *et al.*, 1997) found no overall significant association. However, Germany, with an

average 20 % lower Se concentration than the other countries, demonstrated that men with low toenail Se levels were at increased risk of myocardial infarction. The disparity between studies may also be explained to some extent by the status of other antioxidants such as vitamin E, which may compensate for a deficiency in Se in protection against atherosclerosis. Until results are available from controlled trials such as SUVIMAX (Hercberg *et al.*, 1998), any effects of Se on cardiovascular disease remain unsubstantiated.

From 1984 up until 1990, Finland embarked on a nationwide Se supplementation programme in the form of Se-supplemented fertilizers. The plasma Se levels in the Finnish population were very low in the 1970s and the prevalence of cancer and heart disease was high. In 1986 the Se intake in Finland was 80-100 μg/day, which is 3 - 4 fold higher than intakes in the mid 1970s (Varo *et al.*, 1988). In 1970, the Se content of plasma in Finland was between 0.63-0.76 μM and has risen to 1.40 μM (Varo *et al.*, 1994). During the 1980s the decline in mortality from ischemic events was almost linear, and the Se supplementation programme did not alter this. Thus the improvement in mortality rates from ischemic heart disease could not be attributed to increased dietary Se, and was likely the result of other factors such as increased exercise, reduction in smoking, better health care and improved diets (Varo *et al.*, 1994).

1.6.2 Selenium and endothelial dysfunction

Se-deficiency is associated with an increased severity of atherosclerosis in experimental animals, e.g. a 37 % decrease was measured in the percentage of aortic intima covered by atherosclerotic lesions in hypercholesterolemic rabbits supplemented with Se compared to controls (Wójcicki *et al.*, 1991). Formation of $ONOO^-$ may contribute to endothelial dysfunction (section 1.5.2). The GPX's and SelP in human plasma act as peroxynitrite reductases (Arteel *et al.*, 1999b; Sies and Arteel, 2000; Sies *et al.*, 1997). The TR/Trx system also has peroxynitrite reductase activity (Arteel *et al.*, 1999a; Sies and Arteel, 2000). Impaired endothelium-dependent vasodilation and increased oxidative stress is seen in GPX (-/-) knockout mice (Forgione *et al.*, 2002).

Se supplementation has beneficial effects on several different aspects of endothelial dysfunction. For example, Se supplementation enhances endothelium-dependent relaxation in response to acetylcholine in rat aortic rings (Lu *et al.*, 1994). The mechanism may be through increased $NO^\bullet$ production mediated either directly by Se or through selenoproteins. Se may also exert other effects in the endothelium, including the regulation of prostacyclin and platelet activating factor (Hampel *et al.*, 1989). Neutrophil adherence to bovine mammary artery EC in response to TNFα is increased in cells cultured in Se-deficient media compared to those cultured in Se-sufficient media (Maddox *et al.*, 1999).

Table 1.07 The association of cardiovascular disease mortality and incidence with low serum Se in prospective case-control studies

Study reference	Study population	Major findings
(Salonen *et al.*, 1982)	Case-control study with 283 cases, men and women aged 35-59 yrs, 7 yr follow-up	Serum Se below 45 μg /L was associated with the increased risk of death by CHD and CVD, as well as the risk of fatal and non-fatal MI
(Miettinen *et al.*, 1983)	Case-control study of 33 middle-aged male patients with 1 or more risk factors for CHD 5-7 yr follow-up	Serum Se (50-105 μg /L) was not associated with the development of clinical CHD
(Virtamo *et al.*, 1985)	Cohort study of 1110 male subjects aged between 55-74 yrs 5 yr follow-up	Serum Se below 45 μg /L was negatively correlated to CVD death but not in subjects initially free of CHD No significant association between low serum Se (< 45 μg /L) and the risk of coronary death or MI
(Salonen *et al.*, 1985)	Case-control study of 92 subjects, men and women aged 30-64 yrs 5 yr follow-up	No significant association between low serum Se (< 45 μg /L) and the risk of death from CAD
The Tromsø Heart Study (Ringstad *et al.*, 1987)	Case-control study of 59 male subjects initially free of disease, aged 28-54 yrs, 6 yr follow-up	Low serum Se is not associated with an excess risk of MI
(Kok *et al.*, 1987)	Case-control study of 84 subjects, men and women aged 37-87 yrs, 6-9 yr follow-up	No significant association between low serum Se (< 105 μg /L) and the risk of subsequent death from CVD or CHD
Copenhagen Male Study (Suadicani *et al.*, 1992)	Case-control study of 107 males aged 53-74 yrs, 3 yr follow-up	Individuals whose Se level was below 1 μmol/L had an increased risk in ischemic heart disease
US Physicians Health Study (Simonetta *et al.*, 1995)	Case-control study on 251 male subjects aged between 40-84 yrs, 4-6 yr follow-up	No association between serum Se and risk of myocardial infarction

Abbreviations: CHD, coronary heart disease; CVD, cardiovascular disease; MI, myocardial infarction

1.7 THE SKIN

1.7.1 Structure and function

The skin is directly exposed to a prooxidant environment, including ultraviolet (UV) radiation, ambient levels of O_2, and air pollutants such as sulphur dioxide (SO_2), ozone (O_3) and nitrogen dioxide (NO_2) (Halliwell and Gutteridge, 1999). The important role of ROS in UV-induced skin damage is well documented. UV-induced skin damage includes acute reactions, such as erythema, oedema, followed by exfoliation, tanning and epidermal thickening (Diffey, 1998). Premature skin ageing ('photoaging') and carcinogenesis are believed to be consequences of chronic UV exposure. The skin provides the first line of defence against oxidative damage induced by environmental factors.

Skin tissue consists of numerous cell layers that may be anatomically divided into the cellular epidermis, fibroelastic connective tissue of the dermis, and the underlying subcutaneous fat layer. The epidermis, the outermost skin tissue layer, consists of stratified squamous epithelial cells, separated from the dermis by a basal lamina (figure 1.11).

The epidermis is mainly composed of keratinocytes, which are rich in enzymes such as superoxide dismutase, catalase, TR, and glutathione reductase, and in low molecular mass anti-oxidant molecules such as tocopherol, glutathione and ascorbic acid, and thus provides an efficient protective system against ROS (Maccarrone *et al.*, 1997). Keratinocytes constitute 92 % of the cells in the epidermis forming a stratified multi-layered epithelium. Keratinocytes provide the physical barrier properties of the epidermis and accomplish its repair and regeneration. The keratinocytes situated adjacent to the basement membrane are predominately undifferentiated, rapidly proliferating stem cells. The cells mature as they detach from the basal layer by successive divisions, lose their capacity to proliferate, and undergo keratinisation, becoming more flattened as they approach the surface. Different stratified layers of the epidermis correspond to progressive stages of differentiation. The names of the layers in order of increasing differentiation are the stratum germinativum (basale), stratum spinosum, stratum granulosum, and stratum corneum. The stratum corneum consists of dead, terminally differentiated keratinocytes; the keratinocytes of all other layers are viable.

In addition to keratinocytes, the other components of the skin include melanocytes, Langerhans' cells, leukocytes, mast cells, fibroblasts, and endothelial cells.

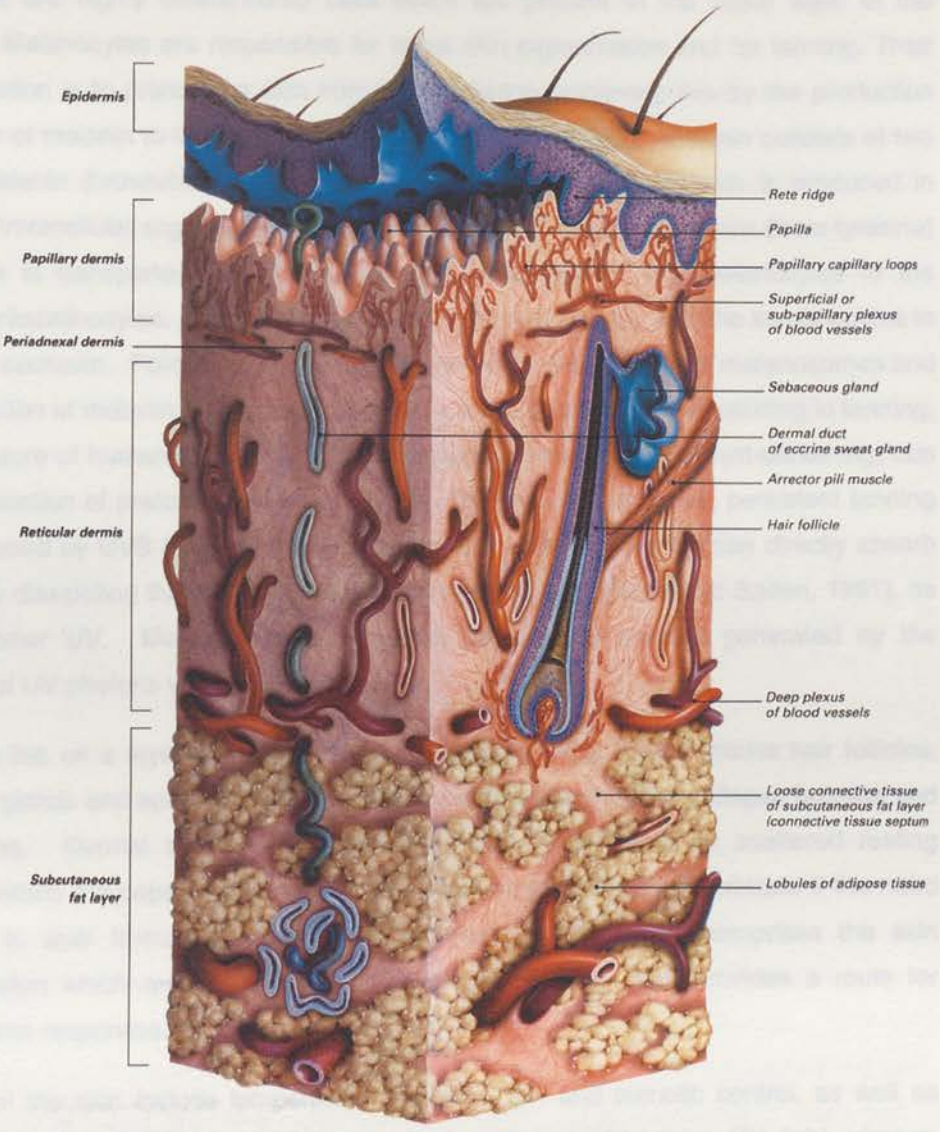


Figure 1.11 Diagrammatic representation of the architecture of human skin, and penetration of UVA and UVB (adapted from (Geras, 1990)). The skin consists of many cell layers that may be anatomically divided into the cellular epidermis and underlying fibroelastic dermis. The epidermis consists of stratified squamous epithelial cells, separated from the dermis by a basal lamina. Keratinocytes make up 92 % of the cells in the epidermis and provide the physical barrier properties of the epidermis, and accomplish its repair and regeneration. Most of the UVB reaching the skin is absorbed by the epidermis, causing epidermal cell damage, while UVA primarily causes dermal injury.

Melanocytes are highly differentiated cells which are present in the basal layer of the epidermis. Melanocytes are responsible for basal skin pigmentation and for tanning. Their primary function is to protect the skin from UV irradiation, achieving this by the production and transfer of melanin to keratinocytes (Gilchrest and Eller, 1999). Melanin consists of two types, eumelanin (brown/black) and phaeomelanin (red/brown). Melanin is produced in specialised intracellular organelles called melanosomes. Following synthesis (from tyrosine) the melanin is transported through the dendritic processes of the melanocytes to the surrounding keratinocytes. The melanin pigment is carried upwards with the keratinocytes to the stratum corneum. Following exposure to UV radiation, the number of melanosomes and their production of melanin greatly increases (Yaar and Gilchrest, 1991), resulting in tanning. Acute exposure of human skin to UV irradiation induces immediate pigment-darkening, due to photo-oxidation of preformed melanin by UVA, followed by a delayed, persistent tanning reaction caused by UVB (Ortonne, 1990; Porges *et al.*, 1988). Melanin can directly absorb UV, thereby dissipating the otherwise injurious energy as heat (Soter and Baden, 1991), as well as scatter UV. Melanin can also absorb free radical species generated by the interaction of UV photons with cellular lipids.

The dermis lies on a layer of subcutaneous fatty tissue. This layer contains hair follicles, sebaceous glands and apocrine (sweat) glands, and consists of dense collagen bundles and elastin fibres. Dermal collagen is capable of being replenished by scattered resting fibrocytes, which are capable of activation into collagen-synthesizing fibroblasts if the need arises, as in scar formation. A network of small blood vessels comprises the skin microcirculation which maintains tissue nutrition, gas tensions, and provides a route for immunological responses.

Functions of the skin include temperature regulation, pH and osmotic control, as well as offering mechanical protection (e.g. insect bites), and protection from UV light, viruses, bacteria, fungi, and thermal stimuli. The skin also provides a nociceptory function. It is the largest organ of the body, being both active and multifunctional. Human skin also produces hormones which are released into the circulation, and which are important for functions of the whole organism. Human skin cells produce insulin-like growth factors, proprio-melanocortin derivatives, neuropeptides, catecholamines (adrenaline and noradrenaline), steroid hormones and vitamin D (from cholesterol), retinoids (from dietary carotenoids), and eicosanoids (from fatty acids) (Zouboulis, 2000).

1.8 ULTRAVIOLET RADIATION AND THE SKIN

1.8.1 Ultraviolet radiation - an introduction

In the ultraviolet (UV) region of the solar spectrum (figure 1.12), only UVA (320 – 400 nm) and UVB (290 - 320 nm) reach the earth's surface. The UVC component (100 -290 nm),

which is even more damaging, is completely filtered out by the ozone (O_3) layer of the stratosphere. UVB is also filtered out to some extent by the ozone layer. However, depletion of this ozone layer, by photochemical reactions involving chlorofluorocarbons, has resulted in more UVB reaching the surface of the earth, with the corresponding increase in photochemical damage to organisms including man (Coldiron, 1992; de Gruijl and Leun, 2000; Slaper *et al.*, 1996). It has been estimated that each 5 % depletion of stratospheric O_3 will raise UVB flux at ground level by 10 %. Ultraviolet light is likely to be the largest toxic insult that the skin receives. Chronic exposure to sunlight is postulated to be a major risk factor for basal- and squamous-cell carcinomas, and malignant melanoma (de Gruijl, 1999). Optical penetration into the skin is governed by a combination of scattering and absorption, but only the latter promotes specific chemical reactions. In the normal epidermis, absorption is the dominant process across most of the optical spectrum.

Different wavelengths of solar radiation have different biological effects. The time course and reaction pattern of UV-mediated sunburn (an acute inflammatory reaction) is wavelength-dependent, as are the effects of UV on cell-mediated immunity and photocarcinogenesis (Fuchs, 1998). This may be accounted for by differences in penetration of UV into the different skin layers (UVA >UVB >UVC), and/or from particular reaction cascades initiated by a specific wavelength (figure 1.13). Most of the UVB is absorbed in the epidermis, causing epidermal cell injury, while UVA causes mainly dermal injury (Pearse *et al.*, 1987). Up to 10 % of UVB light falling on the skin can penetrate through the epidermis to the dermis (Halliwell and Gutteridge, 1999). UVB is much more damaging to the skin than UVA if equal exposures are carried out, but the deeper penetration of UVA and its greater abundance in sunlight suggest that UVA is a major contributor to photodamage. Generally, UVB acts mainly in the epidermis, while UVA injury causes necrosis of the endothelial cells, damaging dermal blood vessels (Clydesdale *et al.*, 2001). UVA comprises a major part of the spectral output of tanning lamps, which are becoming increasingly popular (Freeman *et al.*, 1987).

UVA light is the main source of photo-oxidative stress in skin, but UVB also has an oxidative element (Darr and Fridovich, 1994). Some studies have suggested that UVA-induced cytotoxicity may be just as important as cytotoxicity mediated by UVB (Tyrrell and Pidoux, 1987). Humans are exposed to much less UVB irradiation in comparison to UVA since summer daylight comprises ~ 5 % UVB and 95 % UVA (Obermüller-Jevic *et al.*, 2001). However, many biological effects of exposure to UV are much greater at shorter wavelengths, and UVA radiation is predicted to contribute only 20 % to the harmful effects of sun exposure (Diffey, 1998).

Generation of ROS by UV Exposure

Direct evidence for free radical formation in skin has been obtained by EPR spectroscopy and Fourier Transform Raman spectroscopy (FT-Raman). UV exposure of human and animal skin induced formation of EPR signals, indicating formation of ROS (Nishi *et al.*, 1991; Pathak and Stratton, 1968).

Some of these free radical species are derived from melanin (Chedekel and Zeise, 1988; Collins *et al.*, 1995) (see further information below). Irradiation of human skin with UVB demonstrated a dose-dependent production of H_2O_2 (in the mM range) as measured by FT-Raman (Schallreuter and Wood, 2001). Indirect evidence for the formation of ROS ($OH^{\bullet}$,$O_2^{\bullet-}$, $RO_2^{\bullet}$) by UV irradiation is supplied by spin trapping studies in human (Jurkiewicz and Buettner, 1996) and animal skin (Jurkiewicz and Buettner, 1994; Jurkiewicz and Buettner, 1996; Taira *et al.*, 1992).

Chronic exposure of human skin to increasing doses of UV radiation causes erythema, chronic hyperplasia, mutation, accelerated photo-ageing and photocarcinogenesis. Damage to the skin by UV light produces the characteristic inflammatory response, including recruitment of free radical-generating ($O_2^{\bullet-}$, H_2O_2, HOCl and possibly $NO^{\bullet}$) neutrophils (Afaq and Mukhtar, 2001; Halliwell and Gutteridge, 1999). Photochemical reactions with UV light produce ROS such as $O_2^{\bullet-}$, peroxide ($O_2^{-=}$) and $OH^{\bullet}$. Increased production of H_2O_2 induced by UVA irradiation has been measured in HaCaT cells using fluorescent probes (Petersen *et al.*, 2000). Singlet oxygen can be produced in the skin as a result of photosensitization reactions triggered by exogenous sensitizers such as drugs (e.g. nonsteroidal antipyretics and phenothiazines), cosmetics, food additives, plant toxins or by the endogenous porphyrins that amass in some forms of porphyria (Maccarrone *et al.*, 1997; Wlaschek *et al.*, 1995). Numerous sensitizing dyes and pigments (natural and synthetic) are activated by visible light. The pigmentation of human skin also appears to have a role in generation of $O_2^{\bullet-}$ from photosensitizers (natural and synthetic) that are activated by visible light; this property has been harnessed for therapeutic use in the development of agents for photodynamic therapy for *in vivo* eradication of malignant tumours (Krutmann and Morita, 1999). The pigmentation of human skin also appears to have a role in generation of $O_2^{\bullet-}$ and $O_2^{=}$ from molecular oxygen upon irradiation with UV light. For example, pheomelanin extracted from human red hair produces more $O_2^{\bullet-}$ and $O_2^{=}$ upon irradiation with UV light than eumelanin from black hair. However, the UV-absorbing polymers eumelanin and pheomelanin themselves contain intrinsic free radicals which might trap available soluble radicals by coupling reactions (Schallreuter and Wood, 1989).

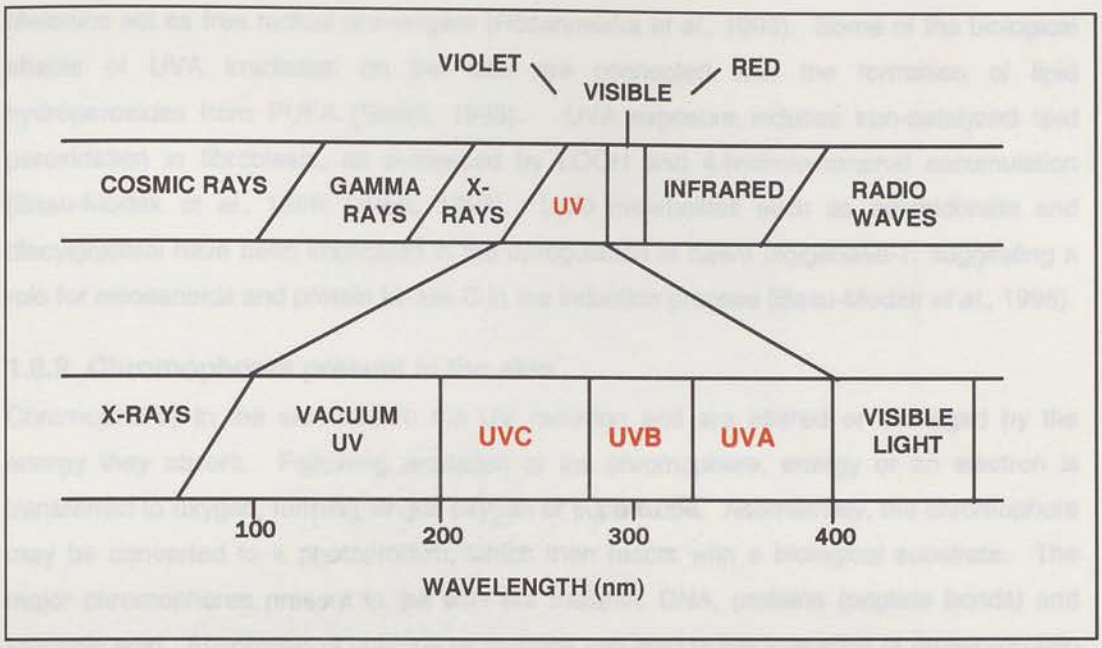


Figure 1.12 The spectrum of electromagnetic radiation (adapted from Soter & Baden, 1991). Solar radiation with wavelengths between 290 and 320 nm (UVB) reaches the earth's surface in relatively small quantities, but these are sufficient to cause sunburn in human skin. Epidemiological evidence strongly suggests that UVB causes most skin cancers in humans. Longer UV wavelengths between 320 and 400 nm (UVA) are also melanogenic and erythemogenic, but the amount of energy required to produce either effect is much greater than for UVB.

Melanins act as free radical scavengers (Rózanowska *et al.*, 1999). Some of the biological effects of UVA irradiation on the skin are connected with the formation of lipid hydroperoxides from PUFA (Girotti, 1998). UVA exposure induces iron-catalyzed lipid peroxidation in fibroblasts, as evidenced by LOOH and 4-hydroxynonenal accumulation (Basu-Modak *et al.*, 1996; Girotti, 1998). Lipid metabolites such as arachidonate and diacylglycerol have been implicated in the upregulation of haem oxygenase-1, suggesting a role for eicosanoids and protein kinase C in the induction process (Basu-Modak *et al.*, 1996).

1.8.2 Chromophores present in the skin

Chromophores in the skin absorb the UV radiation and are altered or damaged by the energy they absorb. Following excitation of the chromophore, energy or an electron is transferred to oxygen, forming singlet oxygen or superoxide. Alternatively, the chromophore may be converted to a photoproduct, which then reacts with a biological substrate. The major chromophores present in the skin are melanin, DNA, proteins (peptide bonds) and urocanic acid. Absorption of photons by proteins can lead to the formation of protein-protein cross-links and protein-DNA cross links. The absorption of UV radiation by proteins and DNA increases dramatically towards the shorter wavelengths in the UVB range, as do the resultant damaging effects on these target molecules (de Gruijl and Forbes, 1995). Constitutive melanin has a broad absorption spectrum across the UVB to visible ranges (290 – 800 nm) (Maccarrone *et al.*, 1997). Melanin is induced by UVB, with a peak at 360 nm.

Urocanic acid (UCA) is synthesized from the essential amino acid histidine using the enzyme histidase. The skin does not contain the enzyme urocanase to catabolize UCA, so the skin contains a high level of accumulated UCA. In the epidermis, UCA exists as a trans-form. Exposure to UV radiation isomerises UCA from the *trans*-form to the *cis*-form, which is thought to be an important mediator of UV-induced regulation of cellular responses in the skin (Duthie *et al.*, 1999).

Absorption of UV photons by DNA molecules produces an excited state that is followed by rearrangement of electrons to form a variety of photoproducts; DNA absorbs UV radiation in the wavebands 230 – 300 nm. DNA absorbs UVB radiation more strongly than UVA (Freeman *et al.*, 1987). The main mechanisms of UV radiation-induced DNA damage are wavelength-dependent. Ultraviolet radiation may damage DNA directly as occurs with shorter wavelengths of UVC or UVB, or indirectly as with longer wavelength UVA, acting via a photosensitiser or through the formation of ROS.

Endogenous, intracellular UVA-absorbing sensitizers in the skin include the tetrapyrroles (e.g. protoporphyrin IX, uroporphyrin III), flavins (FMN, FAD), and reduced pyridine nucleotides (NADH, NADPH) (Girotti, 2001).

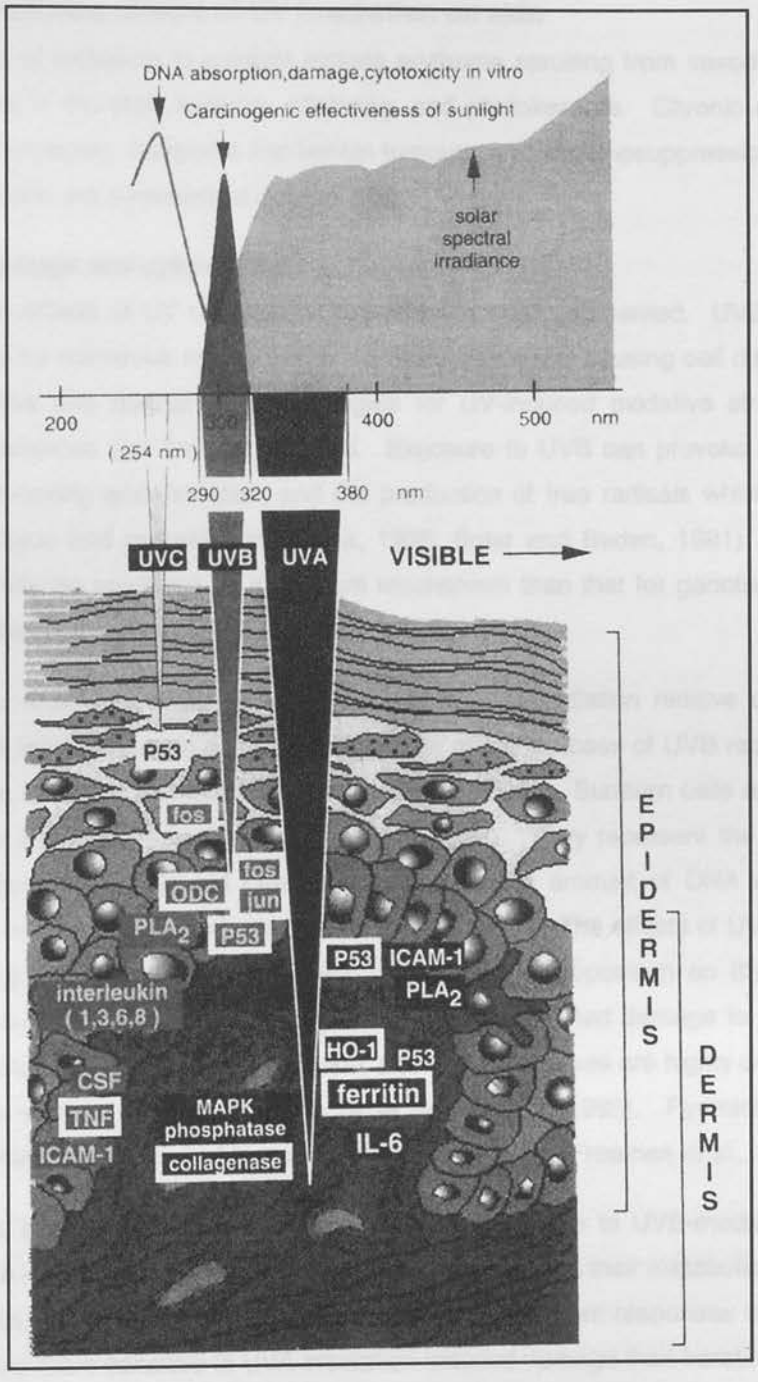


Figure 1.13 The spectrum of solar radiation, and its penetration into the layers of the skin, with resultant activation of various genes. From (Tyrrell, 1995). The longer the wavelength of light, the deeper into the skin it can penetrate. From 2 % to 10 % of UVB radiation will reach the basal layer of the epidermis, whilst 20 % of UVA radiation will penetrate to this depth. Genes which have been demonstrated to be up-regulated by UV irradiation, which include ferritin and collagenase, are marked by a surrounding white box. Abbreviations: ODC, ornithine decarboxylase; PLA_2, phospholipase 2; CSF, colony stimulating factor; TNF, tumour necrosis factor; ICAM-1, intracellular adhesion molecule 1; HO-1, haem oxygenase 1; MAPK phosphatase, mitogen activated protein kinase phosphatase.

1.8.3 Deleterious effects of UV Irradiation on skin

Acute effects of exposure to sunlight include erythema resulting from vasodilatation of the blood vessels in the skin, sunburn, blistering, and photokeratitis. Chronic effects include premature photoaging, malignant and benign tumours, and immunosuppression. The effects of UV on the skin are summarised in table 1.08.

Oxidative damage and cytotoxicity

The cytotoxic effects of UV radiation on the skin are well documented. UVB radiation can damage cells by numerous means and is far more efficient at causing cell death than UVA. Both epidermal and dermal cells are targets for UV-induced oxidative stress, and their antioxidant defences can be overwhelmed. Exposure to UVB can provoke DNA damage, protein cross-linking and oxidation, and the production of free radicals which can damage DNA and induce lipid peroxidation (Fuchs, 1998; Soter and Baden, 1991). UVB-induced cytotoxicity may be mediated by a different mechanism than that for genotoxicity including single strand breaks in DNA (Sugiyama *et al.*, 1992).

Cells *in vitro* which are exposed to high doses of UVB radiation receive a high level of damage, and exhibit necrotic cell death. However at lower doses of UVB radiation cultured cells undergo apoptotic cell death (Mammone *et al.*, 2000). Sunburn cells are dyskeratotic cells induced by acute exposure to UV (Young, 1998). They represent the morphological ultimate stage of apoptotic cell death induced when the amount of DNA damage is too extensive to be repaired (Bayerl *et al.*, 1995; Young, 1987). The effects of UVB to DNA may be direct (e.g. pyrimidine dimer formation due to energy deposition on thymine=thymine bonds) or may be mediated by ROS. Indirect, radical-mediated damage to DNA becomes relatively more important with UVA radiation, and such processes are highly dependent upon other factors such as oxygen level (de Gruijl and Forbes, 1995). Pyrimidine dimers are induced in DNA by irradiation of human skin *in situ* with UVA (Freeman *et al.*, 1987).

Melanocytes, keratinocytes and fibroblasts are all susceptible to UVB-mediated cell death (Dissanayake *et al.*, 1993). Based on their anatomical site and their metabolic and functional characteristics, keratinocytes and fibroblasts may have different responses to UV radiation. Fibroblasts are more sensitive to UVA irradiation-induced damage than keratinocytes (Leccia *et al.*, 1998; Moysan *et al.*, 1995a). PK have lower cyGPX than fibroblasts, but similar levels of GSH, SOD and catalase (Moysan *et al.*, 1995a). Applegate *et al.* attributed the difference in sensitivity of keratinocytes and fibroblasts to UV-induced damage to a higher activity of HO and ferritin in keratinocytes (Applegate and Frenk, 1995; Applegate *et al.*, 1995).

Activation of transcription factors, metalloproteinases and other factors

UVA or UVB can induce activation of a wide range of transcription factors in skin cells, including AP-1 and NF-κB (Tyrrell, 1995). UVB also induces synthesis of metalloprotease enzymes, which can degrade dermal interstitial collagen and other connective tissue components (Brenneisen *et al.*, 1998; Wlaschek *et al.*, 1995). Induction of these enzymes may be mediated by NF-κB and AP-1, whose activation may be promoted by ROS.

Human keratinocytes produce $NO^{\bullet}$ (Heck *et al.*, 1992) generated by iNOS, the calcium-independent inducible isoform of NOS, in response to UVA and UVB irradiation (Deliconstantinos *et al.*, 1995). $NO^{\bullet}$ may participate in the deleterious effect of UV irradiation in conjunction with other ROS produced during the photo-oxidation of intracellular components (Didier *et al.*, 1999), but may also play a role in both UVA- and UVB-induced melanogenesis (Roméro-Graillet *et al.*, 1997).

UV exposure increases the expression of integrins (Neitmann *et al.*, 1999), and induces synthesis of ICAM-1 (a known binding site for leukocyte function) and several cytokines, including interleukin-1 (IL-1), IL-6, IL-8, IL-10, IL-12 and tumour necrosis factor alpha (TNFα). Induction of apoptosis, and formation of sunburn cells may involve TNFα (Schwarz *et al.*, 1995). When the skin is exposed to UV radiation there is a dose-dependent increase in the release of arachidonic acid from membrane bound phospholipids through the activation of phospholipase A_2 and phospholipase C (De Leo *et al.*, 1984). The subsequent increase in prostaglandin is thought to be involved in the erythema reaction following exposure to UV.

Transition metal ion mobilisation

Mobilisation of iron ions following UV exposure makes them available for participation in catalysis of ROS, which may be a mechanism for oxidative skin damage (Trenam *et al.*, 1992). UVA causes release of free iron in skin fibroblasts via proteolysis of ferritin (Pourzand *et al.*, 1999). Chronic exposure of hairless mice to low levels of UVB increases the iron content of the skin (Bissett *et al.*, 1991). Human skin chronically exposed to UV accumulates iron (Jurkiewicz and Buettner, 1994) and has an increased ferritin content when compared with unexposed areas (Morlière *et al.*, 1997). The ferritin content of keratinocytes, which are exposed to more UV than fibroblasts, is much higher when compared with fibroblasts (Applegate and Frenk, 1995). Topical application of the iron chelator Desferal to human skin decreases ROS production by ~ 50 % (Jurkiewicz and Buettner, 1996). Amino acid-based iron chelators reduce UVB-induced oxidative stress in murine dermal fibroblasts (Kitazawa and Iwasaki, 1999). In iron-loaded human fibroblasts, UVA photocytotoxicity increases in a dose-dependent manner with the iron load (Morlière *et al.*, 1997).

Table 1.08 A summary of the effects of UV irradiation on the skin

Effects of UV irradiation on the skin
• Vasodilation of cutaneous blood vessels, resulting in erythema (prostaglandin release)
• Increase in epidermal and stratum corneum thickness
• Increased $NO^{\bullet}$, histamine and PGE_2 production and release from keratinocytes, leading to increased vascular permeability
• Direct cytotoxic damage to epidermal cells; decrease in keratinosomes; formation of dyskeratotic (sunburn) cells; intracellular oedema
• Penetration to the dermis (10 – 15 % of UVB), leading to a direct effect on microvascular EC
• Initiation, promotion and progression of photocarcinogenesis
• DNA damage, leading to lesions (pyrimidine dimers; thymidine dimers; pyrimidine-pyrimidone photoproducts), and oxidative damage leading to 8-hydroxyguanine formation
• Increase in the proto-oncogene *c-fos*, and induction of mutations in the p53 anti-oncogene
• Induction of nuclear factor κB (NFκB), leading to production of pro-inflammatory cytokines IL-1, IL-6 and TNF-α
• Induction of IL-8, which is a chemoattractant for neutrophils, and IL-10, which mediates systemic immune suppression
• Increased expression of ICAM-1 on keratinocytes
• Modification of integrin expression on melanocytes
• Induction of protective metallothionein genes in keratinocytes and fibroblasts
• Induction of metalloproteases which can degrade collagen and other tissue components
• Release of transition metal ions, leading to ROS formation
• Immunosuppression: depletion of immunocompetent cells, and release of immunosuppressive mediators (IL-10, C-UCA, PGE_2)
• Modulation of antioxidants, depleting and inactivating some (GPX; GSH; SOD; GR; α-toc; ascorbate; ubiquinol/ubiquinone; CAT; β-carotene) , and inducing others (ferritin; HO; SOD; Trx and TR)

Abbreviations: CAT, catalase; C-UCA, *cis*-urocanic acid; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HO, Haem oxygenase; α-toc, α-tocopherol; TR, thioredoxin reductase; Trx, thioredoxin; TNF-α, tumour necrosis factor-alpha; PGE_2, prostaglandin E_2

Immune suppression

In addition to causing neoplastic changes, UVB irradiation also causes systemic immune suppression which may impair defence reactions permitting abnormal cells to proliferate and develop into skin tumours. The immunosuppressive events induced by UVB radiation are mediated by several pathways involving DNA damage, photoisomerisation of urocanic acid, or cell membrane damage, and release of interleukin-10 and TNF-α from degranulated mast cells (Black *et al.*, 1997; Clydesdale *et al.*, 2001; Duthie *et al.*, 1999; Kripke *et al.*, 1992; McKenzie and Sauder, 1994).

Antioxidant inactivation

Small molecular antioxidants and antioxidant enzymes can be directly depleted or inactivated by UV in cell-free systems. This has been demonstrated for α-tocopherol (Boguth and Niemann, 1971; Mehlhorn *et al.*, 1990), ascorbate (Bors and Buettner, 1997), and catalase (Cheng and Packer, 1979; Feierabend and Engel, 1986; Zigman *et al.*, 1996). Moreover, ROS produced by UV can directly use up small molecular antioxidants and inhibit enzymic antioxidants in cell-free systems. Glutathione reductase (Tabatabaie and Floyd, 1994), GPX (Blum and Fridovich, 1985), SOD (Bray *et al.*, 1974; Pigeolet *et al.*, 1990; Whiteside and Hassan, 1988), and catalase (Kono and Fridovich, 1982; Sun and Oberley, 1989; Whiteside and Hassan, 1988) are inactivated by ROS. Irradiation of skin cells and murine skin also causes inactivation of antioxidant enzymes and depletion of non-enzymic antioxidants immediately following irradiation; ascorbate (Jurkiewicz and Buettner, 1994; Jurkiewicz and Buettner, 1996; Kagan *et al.*, 1992), α-tocopherol (Fuchs *et al.*, 1989a; Kagan *et al.*, 1992; Shindo *et al.*, 1993), retinol (vitamin A) (Sorg *et al.*, 2002), GSH (Emonet *et al.*, 1997; Fuchs *et al.*, 1989a; Shindo *et al.*, 1993), and ubiquinol/ubiquinone (Fuchs *et al.*, 1989a; Shindo *et al.*, 1993) are depleted, whilst catalase (Miyachi *et al.*, 1987; Pence and Naylor, 1990; Punnonen *et al.*, 1991), GPX (Maisuradze *et al.*, 1987), and glutathione reductase (Fuchs *et al.*, 1989a; Maisuradze *et al.*, 1987) are inactivated. The immediate antioxidant inhibition is of a higher magnitude in the epidermis than the dermis (Shindo *et al.*, 1993). UV exposure can also deplete the levels of α–tocopherol (Thiele *et al.*, 1998), ß-carotene and lycopene (Ribaya-Mercado *et al.*, 1995) in human skin.

However, some studies have shown GPX activity (Fuchs *et al.*, 1989a; Moysan *et al.*, 1993; Shindo and Hashimoto, 1997; Sugiyama *et al.*, 1992), SOD activity (Fuchs *et al.*, 1989a), or GR activity (Shindo and Hashimoto, 1997) to be resistant to inactivation by UV, and catalase to increase rather than decrease upon irradiation (Afaq and Mukhtar, 2001). These disparities may be due to different doses of UV, varying times between irradiation and assay, or differing irradiation protocols (single exposure versus repeated, multiple exposures).

Increases in catalase activity may be due to UV-induced infiltration of leukocytes in the epidermis (Afaq and Mukhtar, 2001).

Carcinogenesis

UVB radiation is a major cause of human skin cancer (de Gruijl, 1999). Natural sunlight is a complete human skin carcinogen, affecting all three stages of photocarcinogenesis: initiation, promotion and progression (Black *et al.*, 1997; McKenzie and Sauder, 1994). The peak of UV-induced carcinogenicity lies within the UVB portion of the spectrum (de Gruijl, 1996). There is increasing evidence to link DNA damage to skin carcinogenesis, the most compelling evidence being that patients with the genetic disorder xeroderma pigmentosum are 2000 times more likely than unaffected individuals to develop skin carcinomas (Brash *et al.*, 1991; McKenzie and Sauder, 1994). Patients with xeroderma pigmentosum are characteristically defective in the mechanisms to repair direct DNA damage; such defects are proposed to predispose cells to a higher frequency of mutation, which may lead to induction of carcinogenesis (Soter and Baden, 1991).

Since UV radiation is a potent DNA-damaging and mutagenic agent, it can initiate carcinogenesis by damaging DNA and inducing mutations in one or more genes (Black *et al.*, 1997). The importance of UVB as an initiator of tumourogenesis is thought to result from its ability to cause mutations in genes controlling the cell cycle, e.g. proto-oncogenes, such as ras, and tumour suppressor genes, such as p53 (Burns and El-Deiry, 1999; Pruitt and Der, 2001), allowing aberrant cells to proliferate, and ultimately to local tumour invasion. Cells in the epidermis accumulate mutations over years of exposure to sunlight and the resulting dysfunctional genes can eventually lead to a malignant transformation. The p53 tumour suppressor gene is mutated in the majority of human cancers, including basal cell carcinoma and squamous cell carcinoma (Brash *et al.*, 1991). Most of the mutations in p53 are located in the DNA binding region of the protein, which contains cysteine residues important for its function. Once activated, p53 can directly bind to single stranded DNA and interact with the DNA replication machinery to regulate the transcription of genes involved in cell cycle arrest (Liu and Pelling, 1995) and apoptosis (Polyak *et al.*, 1997). Confirmation that the P53 tumour suppressor gene is a target in UV carcinogenesis has been confirmed in mouse experiments (Dumaz *et al.*, 1997; van Kranen *et al.*, 1995).

If the intensity of UV radiation is low, then there is limited DNA damage, expression of p53 in the nucleus is increased and activates cell cycle arrest (late G1 and G2/m), apoptosis and nucleotide excision repair (de Gruijl *et al.*, 2001). The cell is then allowed to advance through the cell cycle. If the UV intensity is high then a considerable level of DNA damage occurs, and p53 signals the cell to undergo apoptosis, which will prevent the mutated DNA from being passed on to subsequent generations of daughter cells. The mutated p53 is

thought to have an extended half-life, preventing cells from halting in cycle arrest (to repair their DNA) and proceeding through apoptosis (Liu *et al.*, 1994). Section 1.9 details the epidemiology of skin cancers.

Induction of cell death pathways

Cell death in skin cells can occur by one of three paths, termed necrosis, apoptosis, and terminal differentiation. UVB irradiation can induce both the apoptosis and necrosis pathways in normal human keratinocytes. Necrotic cell death is caused by a period of acute and severe physical trauma to a cell, resulting in the disruption of cell organelles and membrane integrity, and ultimately cell death. Necrosis is characterized by random DNA fragmentation, cell swelling and lysis.

Apoptosis, in comparison, is an active form of cell death with specific morphological features which differ from necrosis (Kulms and Schwarz, 2000). Apoptotic death is characterised by cell shrinkage, plasma membrane blebbing, condensation of chromatin, protease and endonuclease activation, fragmentation of the nucleus, and cell fragmentation into apoptotic bodies that are phagocytosed by neighbouring cells (Kulms and Schwarz, 2000). As the cells are removed quickly and do not lyse, no cellular contents leak and there is no release of inflammatory mediators, unlike necrotic cell death. However, in the absence of macrophages, apoptotic cells will eventually necrose.

The extent of peroxidative injury in a photodynamically-stressed cell may ultimately determine whether it survives or yields to apoptosis or necrosis (Girotti, 1998). The possible outcomes may be viewed as a graded pattern of responses to membrane damage that increases in the proceeding order: (a) no net damage when constitutive antioxidant capacity is sufficient to prevent or repair peroxidative damage; (b) sublethal/mild oxidative damage, possibly triggering induction of antioxidant proteins for enhanced cytoprotection; (c) more extensive damage, which triggers apoptosis, beyond which any constitutive or inducible antioxidant protection is overwhelmed; and (d) very extensive damage with membrane lysis, which abolishes any programmed cell death response, and leads to necrotic cell death. When normal human skin is exposed to UVB, some of the keratinocytes in the epidermal layer develop into apoptotic cells, referred to as sunburn cells. Low doses of UVB (5-20 mJ/cm^2) induce apoptosis, whereas increasing doses (>20 mJ/cm^2) of UVB produce direct necrosis in HaCaT cells (Mammone *et al.*, 2000). Such a threshold of stimulus for necrotic cell death has also been demonstrated for H_2O_2 (Lennon *et al.*, 1991).

Many studies have reported that mammalian cells overexpress particular cytoprotective enzymes in response to sub-lethal oxidative stress. Haem-oxygenase-1 (HO-1) and ferritin are induced in fibroblasts irradiated with UVA (Basu-Modak *et al.*, 1996; Vile *et al.*, 1994; Vile

and Tyrrell, 1993). Such induction is though to be a defensive tactic against haem- and iron-amplified oxidative damage triggered by UVA-generated singlet oxygen.

1.8.4 Cellular signalling in the skin in response to UV

NF-κB is an oxidative stress-sensitive transcriptional activator which activates multiple target genes, many of which have proinflammatory effects in common (Rahman and MacNee, 2000). In human keratinocytes, a variety of stimuli, including ozone, UVA and UVB, and cytokines activate NF-κB (Fuchs, 1998; Tyrrell, 1995). Induction of NF-κB is an early response to oxidative stress, and the NF-κB signalling pathway appears to be a natural protective mechanism against injury. Certain proteins related to oxidative stress, such as Mn-SOD (Rahman and MacNee, 2000), inducible NO (Spink *et al.*, 1995), and ferritin (Shi *et al.*, 1994) are induced after activation of NF-κB.

Activation of AP-1 results in the expression of downstream target genes such as γ-glutamyl-cysteine synthetase, glutathione S-transferase, and quinone reductase (Bergelson *et al.*, 1994; Sekhar *et al.*, 1997). AP-1 responsive genes induced by irradiation with UV include genes encoding HO and matrix proteases (Maccarrone *et al.*, 1997; Petersen *et al.*, 1992). Ascorbate may mediate cellular responses to counteract UV-mediated damage and death by adjusting the activity of the AP-1 pathway, and modulating the expression of AP-1-regulated genes in HaCaT and normal human keratinocytes (Catani *et al.*, 2001).

Mitogen-activated protein (MAP) kinases (e.g. extracellular signal-regulated protein kinases (Erks) and c-Jun N-terminal kinases (JNKs)) are tyrosine phosphoproteins which are important mediators of the cellular stress response. MAP kinases are phosphorylated and activated by ROS (Gupta *et al.*, 1999). Distinct signalling cascades involving either oxidative stress or GTPase-coupled pathways are triggered by different forms of cellular stress, implying that antioxidants may only affect MAP kinase activation induced by oxidative stress.

1.9 Epidemiology of skin cancer

It is widely accepted that UV radiation is carcinogenic in humans and can produce photochemical changes in superficial tissues resulting in acute and chronic adverse health effects.

The wavelength dependence (action spectrum) of UV-induced skin cancers has been estimated (de Gruijl, 1996). The UVA contribution to the carcinogenicity of sunlight is approximately 20 %, with UVB causing about 80 %. Factors that influence induction of skin cancer by UV irradiation include physical factors such as dose response, dose rate, dose fractionation, radiation quality (wavelength) (Black *et al.*, 1997), and biological factors such as skin type, genetic constitution, age, and anatomical site.

The incidence of the three main types of skin cancer, basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and cutaneous malignant melanoma (CMM), almost doubled between 1980 and 1990 (Devesa *et al.*, 1995). The number of skin cancer-related deaths in the UK has increased from 1040 cases in 1986 to 1522 cases in 1997 (Murphy, 2002).

Non-melanoma skin cancers (NMSC), comprising BCC and SCC, the most common type of skin tumours to occur, are generally curable, and death is uncommon. The occurrence of NMSC is related to chronic UV exposure and is therefore more common in fair skinned individuals (Stern and Momtaz, 1984), albinos and individuals with *xeroderma pigmentosum.* Some of the apparent increased incidence of skin cancers may be to due to better reporting.

1.9.1 Basal cell carcinoma

BCCs are slow-growing, locally invasive malignant epidermal skin tumours. BCC is the most common type of skin cancer (Diepgen and Mahler, 2002). It is estimated that 4,000 are currently diagnosed annually in the UK (Murphy, 2002). The most significant etiologic factor is chronic exposure to UV radiation; consequently the head and neck are the most commonly involved sites (Armstrong and Kricker, 2001; de Gruijl, 1999). The incidence of BCC rises with increasing age up to middle age (English *et al.*, 1997; Buettner *et al.*, 1998), but such tumours are seldom fatal and metastasize rarely.

1.9.2 Squamous cell carcinoma (SCC)

SCCs are malignant tumours arising from keratinocytes in the epidermis or from mucous membranes. SCCs are also locally invasive and more aggressive than BCCs, which makes them more likely to metastasize. SCCs represent the second most common type of skin cancer in the USA, Australia and Europe (Diepgen and Mahler, 2002).

1.9.3 Cutaneous malignant melanoma (CMM)

CMM are malignant tumours stemming from melanocytes. Although it is less common than BCC or SCC, CMM accounts for the majority of deaths from skin cancer. This is the most aggressive type of skin cancer, and can metastasise very rapidly. The 1980's saw an increase in mortality from malignant melanomas in England and Wales. The incidence and mortality rates of melanoma are now falling in Australia and Hawaii (Rigel, 2002). It is thought that the risk of melanoma may be associated with short periods of intense exposure to UV radiation and possibly acute episodes of sunburn, particularly in childhood (de Gruijl, 1996; Elwood, 1992).

1.10 Selenium, UV and the skin

Se appears to have an important role in protecting the skin from the injurious effects of UVB, and against UV-induced skin cancers (McKenzie, 2000). Se prevents oxidative damage caused by UV, but fails to stop dimer formation, which is the predominant carcinogenic

photoproduct. The production of inflammatory and immunosuppressive cytokines is prevented by Se, which would otherwise impair immune responses following UV exposure. Se also raises cellular and humoral immunity. In several disease states, including melanoma and NMSC (Deffuant *et al.*, 1994), plasma levels of Se are low, but this may be due to an acute phase response with concomitant decrease in selenoproteins in plasma.

Se supplementation in mice substantially decreases the level of skin damage, tumour formation and overall mortality following UVB exposure (Overvad *et al.*, 1985; Pence *et al.*, 1994; Stewart *et al.*, 1996). In man Se-deficiency is associated with up to a 4-fold increase in the risk of developing skin cancer (Clark *et al.*, 1984; Deffuant *et al.*, 1994; Reinhold *et al.*, 1989). Topical application of Se in the form of selenomethionine can protect humans and mice from acute skin damage and decrease sunburn cell formation following exposure to UVB (Burke *et al.*, 1992a; la Ruche and Césarini, 1991; Thorling *et al.*, 1983). In Se-deficiency there is increased lipid peroxidation in UVA-exposed cultured fibroblasts (Moysan *et al.*, 1995b). In cultured murine keratinocytes, sodium selenite decreases UV-induced oxidative damage to DNA when added to the growth medium (Stewart *et al.*, 1996). Sodium selenite prevents oxidative DNA damage to HaCaT cells induced by irradiation with UVA (Petersen *et al.*, 2000). Selenomethionine induces DNA repair enzymes in human fibroblasts exposed to UV (Seo *et al.*, 2002). Incubation with sodium selenite increases GPX activity and decreases UVA-induced lipid peroxidation in human skin fibroblasts (Emonet-Piccardi *et al.*, 1998; Leccia *et al.*, 1993). In addition, dietary supplementation in mice with sodium selenite increases GPX levels and decreases the incidence of chemically-induced skin carcinogenesis (Perchellet *et al.*, 1987). Topically applied thermal water from natural spas containing a high level of Se decreases the level of UVB-induced lipid peroxidation and skin carcinogenesis in hairless mice (Overvad *et al.*, 1985). Similarly, Se supplementation in patients for 14 days diminishes the level of lipid peroxidation induced by exposure to UVB (Pietschmann *et al.*, 1992). Supplementation of primary keratinocytes with Se prevents UVB-induced apoptosis, but does not decrease the levels of UVB-induced p53. This suggests that Se prevents UVB-induced cell death by inhibiting p53-independent pathways (Rafferty *et al.*, 2003a).

Both selenomethionine and sodium selenite can protect keratinocytes (Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b; Stewart *et al.*, 1996) and melanocytes (Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b) from UV-induced cell death at nanomolar concentrations. These studies are described in further detail in chapter 5. Seleno-compounds do not absorb in the UVA/UVB wavelengths (McKenzie, 2000), and so cannot act as sunscreens. Sodium selenite can inhibit caspase-3 (an essential enzyme in the induction of apoptotic pathways) activation *in vitro* (Park *et al.*, 2000). Selenite, unlike other seleno-compounds, does not induce activation of caspase-3 in HL-60 cells, suggesting that selenite-induced cell death could be

due to necrosis rather than apoptosis (Kim *et al.*, 2001b). Caspase-3 also increases oxidative stress, and thereby damage, by decreasing catalase, GPX, and SOD activities (Pence *et al.*, 1994).

UV irradiation is well documented in its immunosuppressive effects (Clydesdale *et al.*, 2001; Duthie *et al.*, 1999). An adequate intake of Se is essential for both humoral (antibody-mediated) and cell-mediated immunity. Se-deficiency impairs synthesis of complement and immunoglobulins (McKenzie *et al.*, 2002b), and causes defective neutrophil function and decreased neutrophil numbers, increases H_2O_2 release during neutrophil phagocytosis, and reduces natural killer cell activity (McKenzie *et al.*, 2001). Conversely, Se supplementation augments host antibody and complement responses to tetanus and typhoid toxins, sheep erythrocytes, and immunoglobulins. Supplementation with Se also increases the number of antibody-producing B-cells, increases T-cell-dependent antibody production (T-cell help), and increases in neutrophils (McKenzie *et al.*, 2001). The immunomodulatory effects of Se occur chiefly through the following mechanisms: (1) anti-inflammatory effects of selenocompounds or selenoproteins; (2) selenocompounds or selenoproteins modulating the redox state of the cell through antioxidant action; (3) through the production of cytostatic and anticancer compounds as products of metabolism of Se. The principal mechanisms stimulated by Se may be summarised as follows: (a) detoxification of organic hydroperoxides and H_2O_2; (b) regulation of the balance of activity in the pathways of eicosanoid synthesis, resulting in preferential synthesis of leukotrienes and prostacyclins over thromboxanes and prostaglandins; (c) down-regulation of the expression of cytokines and adhesion molecules; (d) up-regulation of the expression of interleukin-2, resulting in enhanced lymphocyte, natural killer and lymphocyte activated killer cell activity.

In the skin, Se supplementation protects keratinocytes from UV-induced release of IL-10, which would otherwise inhibit antigen presentation and cell-mediated immune responses. Inhibition of UVB-induction of the proinflammatory cytokines IL-6 and IL-8 has also been shown for Se-supplemented keratinocytes (McKenzie *et al.*, 2002b).

Selenoenzymes in the skin are discussed below in section 1.11.1.

1.10.1 Selenium and skin cancer

The major trial of Se in chemoprevention carried out by Clark *et al.* (described in detail in section 1.2.4) initially used the primary endpoint of NMSC in subjects with a history of at least 2 skin cancers receiving either 200 μg Se or placebo daily (Clark *et al.*, 1996; Clark *et al.*, 1998). The results reported 13 years after the initiation of the trial showed no effect of Se on NMSC, but showed secondary endpoint effects of 50 % lower total cancer mortality and 37 % lower total cancer incidence (section 1.2.4). It is conceivable that this study used too low a dose of Se to achieve the protection required from the damage mediated by UV

irradiation. Although Se can prevent UVB-induced skin tumours in hairless mice (Burke *et al.*, 1992b; Overvad *et al.*, 1985; Pence *et al.*, 1994), the doses of Se used in such studies are often too high to be applicable to the human situation, and definitive evidence of protection against UVB-mediated skin carcinogenesis in humans by Se is lacking. Case-control studies of internal human cancers are difficult to interpret since neoplastic tissue sequesters Se (Clark *et al.*, 1984), but this may not apply in the case of skin cancers.

1.11 Antioxidant systems in the skin

A complex antioxidant defence system has evolved in the skin to protect against ROS. The individual antioxidant enzymes are located in specific subcellular sites and are substrate specific. Several antioxidant defence mechanisms have been identified in skin. Water-soluble antioxidants include ascorbic acid, glutathione, and enzymes such as SOD, catalase, GR and GPX. The individual antioxidants and selenoproteins are described in detail in sections 1.1.5 and 1.2.7, respectively. The epidermal TR/Trx system may be important in an antioxidant defence role (Schallreuter and Wood, 1986; Schallreuter and Wood, 1989; Schallreuter and Wood, 2001). Lipid soluble antioxidants in the skin include α-tocopherol and ubiquinones (Leccia *et al.*, 1998).

Sunlight induces the synthesis of melanin, which has a broad absorbance spectrum that ranges through the UVB, UVA and visible ranges. Eumelanin may be highly protective, but this is not necessarily true of pheomelanin (Halliwell and Gutteridge, 1999). ROS, such as the superoxide anion, can affect tyrosinase activity (Wood *et al.*, 1995). Furthermore, H_2O_2 and UVB have been shown to oxidise BH_4 (tetrahydrobiopterin), an essential cofactor for phenylalanine hydroxylase, to 6 biopterin (Schallreuter *et al.*, 1999; Wood *et al.*, 1995). This oxidation is reversible by TR (Schallreuter and Wood, 2001) (see below).

Whereas high UV doses and ROS levels are known to overwhelm the antioxidant enzymatic defence system, repetitive low-dose UV exposures prevent phototoxicity upon subsequent higher UV doses (Meewes *et al.*, 2001), indirectly suggesting that the skin, apart from other principal protective strategies like inducible melanogenesis, is equipped with an inducible adaptive antioxidant defence mechanism. Up-regulation of antioxidants by UV exposure is described in section 1.11.3.

1.11.1 Antioxidant enzymes in the skin

Catalase

Catalase is thought to be a less important antioxidant enzyme than GPX in the skin, since catalase-deficient fibroblasts do not show decreased viability following exposure to UV compared with fibroblasts exhibiting the enzyme (Shindo and Hashimoto, 1995). However, catalase can decrease the damage to other antioxidant enzymes during chronic UV

exposure (Shindo and Hashimoto, 1995), and when added exogenously to culture media can decrease the formation of sunburn cells in mouse skin explants following exposure to UVB (Miyachi *et al.*, 1983). Following exposure to UVA and UVB the level of catalase present in the skin is greatly reduced (Fuchs *et al.*, 1989a; Pence and Naylor, 1990; Punnonen *et al.*, 1991). The decrease in catalase is probably due to the irreversible oxidative damage of the enzyme.

Superoxide dismutase (SOD)

SOD is present in the skin in both the Cu/Zn (Cu/Zn-SOD) and Mn (Mn-SOD) forms (Carraro and Pathak, 1988; Schallreuter and Wood, 1989). In mice given a single injection of SOD either just before or immediately following UV exposure, formation of sunburn cells was significantly decreased (Danno *et al.*, 1984). The level of SOD in the skin decreases following UVB exposure (Fuchs *et al.*, 1989a; Fuchs *et al.*, 1989b; Miyachi *et al.*, 1987) (see section 1.8.3 above). As seen with catalase, the damage to SOD is probably a result of direct oxidative damage to the enzyme. Such a decrease in SOD activity following UV irradiation can be prevented by treating mice with SOD in liposomes (Miyachi *et al.*, 1987). Human dermal fibroblasts *in vitro* irradiated with single and repetitive low doses of UVA show significant increases in MnSOD protein and mRNA, and this induction offered substantial protection against the cytotoxic effect of UVA exposure (Poswig *et al.*, 1999). When added exogenously to tissue culture media, SOD prevents the formation of sunburn cells (Miyachi *et al.*, 1983). However the H_2O_2 formed by the action of SOD is damaging to cells in itself, so to be protective any increase in SOD should ideally occur in unison with an increase in catalase or GPX.

Peroxiredoxin (Prx)

At least three isoforms of Prx are differentially expressed in rat skin, in the epidermis, hair follicles, and sebaceous glands, where TR and Trx are also present (Lee *et al.*, 2000a); this is expected since Prx can only function when TR and Trx are present as an electron donor system. Expression of at least one Prx can be increased by UV irradiation, suggesting a protective role (Lee *et al.*, 2000a).

Selenoenzymes

In humans, the concentration of Se in the epidermis is higher than in the dermis (Molokhia *et al.*, 1979). The corresponding expression of selenoproteins may reflect this. The epidermis has a significantly higher cyGPX activity than the dermis in human skin (Shindo *et al.*, 1994). In mice, levels of catalase, GPX, glutathione reductase, and GSH (but not SOD) are higher in epidermis than in dermis (Shindo *et al.*, 1993). In human skin there are higher activity levels of catalase, SOD, cyGPX, and GR than in the dermis (Shindo *et al.*, 1994). This presumably reflects the higher level of damaging UV that the epidermis is exposed to.

Keratinocytes express lower levels of TR and PHGPX than melanocytes, but are more resistant to UVB-induced cell death than melanocytes (Rafferty *et al.*, 1998b). TR and PHGPX expression were greater in melanocytes than keratinocytes, but not as great as in fibroblasts. Clearly, the susceptibility of a cell to UVB-mediated damage will be dependent upon an extensive range of enzymatic and non-enzymatic antioxidant systems, and DNA repair mechanisms, rather than selenoproteins in isolation. This is supported by studies showing that keratinocytes have twice the specific activity of GPX of fibroblasts, and that the 50 % toxic dose for cell death by UVA is 48 J/cm^2 for keratinocytes, and only 5.8 J/cm^2 for fibroblasts (Leccia *et al.*, 1998).

Normal skin shows Trx immunohistochemical staining in the sebaceous glands, secretory components of sweat glands, and the outer root sheath of the hair follicle, but not in the interfollicular epidermis (Wakita *et al.*, 1992). TR and Trx are expressed in the hair follicles and basal cell layer of the epidermis in the rat (Lee *et al.*, 2000a). In another report, Trx was present in the hair follicle, sebaceous and sweat glands, but not in normal epidermis (Sachi *et al.*, 1995). However, enhanced expression of Trx has been demonstrated in the epidermal cells of skin exposed to UV (Sachi *et al.*, 1995), and this may be a protective response to oxidative stress insult. UVB irradiation of keratinocytes increases Trx levels in the cytosol, followed by its translocation into the nucleus (Schallreuter *et al.*, 1994b). Such translocation of Trx to the nucleus upon irradiation is also true for UVA-treated human skin fibroblasts (Didier *et al.*, 2001), and for phorbol acetate-treated HeLa cells (Hirota *et al.*, 1997). These data suggest an important role for Trx in the protection of DNA against oxidative damage. Both Trx and TR, and also glutaredoxin, are induced in mouse skin upon application of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a PKC activator and (non-carcinogenic) tumour promoter (Kumar and Holmgren, 1999).

Schallreuter and Wood have proposed that TR is an extremely active free radical reducing enzyme on the plasma membrane (Schallreuter and Wood, 1986). They developed a bioassay for TR: using electron spin resonance spectroscopy (ESR), the decrease in ESR amplitude of a cationic nitroxide (free radical; spin label) is monitored. The spin labelled nitroxide is reduced in the epidermis to an ESR silent product. Schallreuter and Wood suggested that this reduction is specific for TR in epidermis. Reduction of spin label on skin, on keratinocytes, melanocytes and purified *E. coli* TR was inhibited by thioprotein inhibitors, $NADP^+$, anthralin, azelaic acid and other saturated dicarboxylic acids, and Trx (competitive substrate) (Schallreuter *et al.*, 1986; Schallreuter and Wood, 1987). However these studies have been justifiably criticized because of the non-specific nature of the assay employed for measuring TR (Fuchs, 1988) (this is further discussed in section 4.3). Studies by Fuchs *et al.* have suggested that the main reducing activity for piperidinoxyl-type nitroxides in mouse

skin homogenates is ascorbate, and pyrroline-type nitroxides are reduced by a non-ascorbate method, possibly GSH (Fuchs *et al.*, 1989b).

There is a link between reduction of free radicals by TR and melanin biosynthesis by melanocytes (Schallreuter *et al.*, 1994b; Schallreuter and Wood, 1986). Schallreuter and Wood reported a direct correlation between TR activity and skin pigmentation. TR activity in patients with skin type VI (highly pigmented) is 5-fold higher than that found in patients with skin type I (fair skin) (Schallreuter *et al.*, 1987). Tyrosinase is essential to the first 2 steps of melanin synthesis (Yaar and Gilchrest, 1991). Oxidised thioredoxin stimulates, whereas reduced thioredoxin inhibits tyrosinase activity (Schallreuter and Wood, 1989). The oxidation of the cofactor $6BH_4$, by H_2O_2, to cytotoxic 6 biopterin is reversible by TR (Schallreuter *et al.*, 1994a; Schallreuter and Wood, 2001).

1.11.2 Non-enzymatic antioxidants in the skin

Keratinocytes and fibroblasts contain millimolar levels of GSH, ascorbate and DNA repair enzymes. Amongst the photoprotection systems in the skin, the availability of a sufficient supply of reduced GSH is essential (Maccarrone *et al.*, 1997). GR activity is extremely high in the skin, and it is predicted that the amount is sufficient to recycle all cutaneous GSH in under 1 min (Connor and Wheeler, 1987). This apparent excess capacity is an indication of the importance of high GSH levels. At wavelengths corresponding to UVA, GSH is particularly important as the unique hydrogen donor in the quenching of H_2O_2 by GPX (Afaq and Mukhtar, 2001). Cellular GSH also has an important role in regulating HO-1 activity by via thiol/disulphide redox control (Basu-Modak *et al.*, 1996).

α-tocopherol is effective against UV-induced, immunosuppression (Clement-Lacroix *et al.*, 1996; Gensler and Magdaleno, 1991; Yuen and Halliday, 1997), lipid peroxidation (Saral *et al.*, 2002; Yuen and Halliday, 1997), sunburn cell formation (la Ruche and Césarini, 1991), and oxidative DNA damage (Stewart *et al.*, 1996).

In comparison to α-tocopherol, the basal concentration of β-carotene in human keratinocytes is several-fold lower (Shindo *et al.*, 1994; Vahlquist *et al.*, 1982). However, β-carotene accumulation in keratinocytes can reach tissue concentrations far greater than those of α-tocopherol. β-carotene administration is protective against light-induced skin damage in patients with porphyria (Mathews-Roth, 1987). Sunlight depletes β-carotene in the skin, consistent with a protective role in normal subjects (Biesalski *et al.*, 1996).

1.11.3 Antioxidant upregulation by UV exposure

UV radiation may exert opposing effects on the activities of antioxidants in the skin, depending on dose and sequence of radiation exposures. Commonly, high dose acute UV

exposure may inhibit, and low dose chronic UV exposure induce particular antioxidants. Chronic UVA exposure elevates GPX activity in mouse skin (Maeda *et al.*, 1991). In cultured human skin fibroblasts, UVA induces 'heat-shock' proteins, including haem oxygenase-1 (Hsp32/HO-1), co-upregulated with ferritin (Tyrrell, 1995). The induction of HO by UVA is thought to be a protective response to oxidative stress since bilirubin and biliverdin have antioxidant activities (Clark *et al.*, 2000).

Keratinocytes have higher basal levels of haem oxygenase (HO-2), but little inducible (HO-1) activity. This may be explained by the fact that considerable UV radiation reaches the keratinocytes at the base of the epidermis, whereas higher exposures are needed to reach the dermal fibroblasts. Upregulation of HO-1 by UVA has been shown in human fibroblasts however (Basu-Modak *et al.*, 1996).

Upon UVA irradiation of human fibroblasts, the expression of both Trx and TR is increased (Didier *et al.*, 2001). A UV insult to skin increases peroxiredoxin II expression in rat skin within 15 min of irradiation (Lee *et al.*, 2000a). Repetitive and chronic exposure of hairless mice to UVB results in an increase of SOD activity after 36 wk (Okada *et al.*, 1994). In chronically UVB-exposed sun-exposed human skin, SOD activity (Punnonen *et al.*, 1995), tocopherol and ubiquinone concentrations are elevated (de Simone *et al.*, 1987).

1.11.4 Antioxidant photoprotection

Studies in cell culture systems and animals

The protective effects of antioxidants have been displayed in several studies in which they were administered to animals or cells in culture prior to UV irradiation. α-tocopherol, administered in liposomes to human keratinocytes in culture, provided protection from UV radiation-induced cell death (Werninghaus *et al.*, 1991), and protected human skin fibroblasts *in vitro* from UVB-mediated cytotoxicity, while MDA production was unaltered (Kondo *et al.*, 1990). In HaCaT cells, pre-incubation with ascorbate or α-tocopherol protects against UVB-mediated peroxide formation, but cannot protect against UVB-mediated cytotoxicity, suggesting that the mechanism of toxicity may not be mediated by ROS alone (Podhaisky *et al.*, 2000). Chinese hamster V-79 cells incubated with α-tocopherol succinate are protected from UVB-induced cytotoxicity (Sugiyama *et al.*, 1992), but the amount of DNA single strand breaks and induction of chromosomal mutations induced by UVB are not altered, perhaps suggesting that UVB-induced DNA damage does not directly correlate with the cytotoxic mechanism of UVB. Indeed, Se protects against UVB-mediated cytotoxicity (Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b) and oxidative damage to DNA (8-hydroxy-2-deoxyguanosine formation), but not against DNA lesion damage (cyclopyrimidine dimers; excision repair sites) induced by UVB irradiation in keratinocytes (Rafferty, 2000; Rafferty *et al.*, 2003b). Studies utilising Se in photoprotection are described in section 1.10.

Topically applied α-tocopherol solution or β-carotene solution, or ascorbic acid delivered intraperitoneally before UV exposure significantly reduced TBARS formation in mouse skin (Khettab *et al.*, 1988; Kobayashi *et al.*, 1996a; Pugliese and Lampley, 1985). UVB-induced (Jurkiewicz *et al.*, 1995) or UVA-induced (Sorg *et al.*, 2002) formation of lipid-derived radicals was significantly reduced by topical application of α-tocopherol to hairless mice, or guinea pigs (Saral *et al.*, 2002). These results suggest that α-tocopherol protects against UV-induced lipid peroxidation in the skin. Apart from scavenging ROS, α-tocopherol may act as a sunscreen, since it absorbs UV light (with maximum absorption at 295 nm), or a cellular response moderator, for example altering inflammatory cascades. β-carotene absorbs radiation in the near violet and visible spectrum (360 – 550 nm) (Pathak, 1982). Vitamin E also possesses an anti-inflammatory effect via the inhibition of phospholipase.

Murine skin can be protected from UVB-induced erythema by topical application of α-tocopherol or ascorbate prior to exposure to UV (Khettab *et al.*, 1988; Möller *et al.*, 1989; Roshchupkin *et al.*, 1979). Inhibition of photocarcinogenesis in mice has been demonstrated using topical application of α-tocopherol (Gensler and Magdaleno, 1991) or glutathione derivatives (Kobayashi *et al.*, 1996b), as well as dietary α-tocopherol (Gerrish and Gensler, 1993; Pathak, 1982) or β-carotene (Bissett *et al.*, 1990).

Trials of antioxidants in photoprotection in humans

The results of randomised trials of antioxidants in the prevention of UV-induced damage have been largely ineffective, or minimally protective (table 1.09). There is no convincing evidence in the literature that α-tocopherol alone is clinically useful in prevention or treatment of normal skin reactions to UV irradiation (Fuchs, 1998). However, tocopherol is regenerated from the tocopheryl radical by ascorbate (section 1.1.5), so the two dietary components may act synergistically to provide protection. It has been suggested that β-carotene supplementation is unlikely to modify the severity of cutaneous photodamage in normal individuals to any meaningful extent in clinical terms (Garmyn *et al.*, 1995). The effectiveness of systemic ascorbate in protection against sunburn is regarded as poor and questionable (Fuchs, 1998). It remains to be seen whether antioxidants/combinations will prove beneficial in protection against photodamage in human skin.

The ultimate objective in photoprotection is prevention of chronic skin damage, such as skin cancer. The molecular pathways leading to UV-induced inflammation are presumably different to those biochemical cascades resulting in immunosuppression, photo-aging and cancer (Fuchs, 1998). UV-induced erythema may be an inappropriate endpoint and invalid indicator for assessing the efficacy of protection of antioxidants in prevention of photoageing, photoimmunosuppression, and photocarcinogenesis (Fuchs, 1998).

Table 1.09 Trials of Antioxidants as Photoprotective Agents‡

Study	Study Population	Major Findings
(Mathews-Roth *et al.*, 1972)	30 healthy male volunteers; 21 – 49 years of age (180 mg β-carotene/day for 10 weeks)	Small but significant effect in increasing MED with β-carotene ($p < 0.025$)
(Wolf *et al.*, 1988)	23 healthy volunteers (150 mg β-carotene/day for 4 weeks)	No effect of β-carotene on UVA/UVB-induced erythema, or UVB-mediated DNA damage
(Greenberg *et al.*, 1990)	1805 patients with recent non-melanoma skin cancer, followed for 5 years	No effect of beta carotene on either number or time of occurrence of new NMSC
(Msika *et al.*, 1990)	6 volunteers (topical α-tocopherol 1 %)	Topical α-tocopherol reduced sunburn cell formation, but had no effect on UVB/A-induced erythema
(Murray *et al.*, 1991)	10 healthy volunteers (topical 10 % ascorbic acid (w/v) for 5 days)	Topical ascorbate reduced UVB-induced erythema ($p < 0.001$)
(Murray *et al.*, 1992)	10 individuals (topical ascorbate)	Topical ascorbate significantly attenuated UVA-induced immediate pigment response ($p < 0.001$) and increased MED ($p < 0.05$)
(Werninghaus *et al.*, 1994)	12 healthy volunteers; 2 women and 12 men, aged 25 - 84 years (oral α-tocopherol 400 IU/day for 6 months)	No clinical (MED) or histological (sunburn cell number) difference in acute UVB response with α-tocopherol
(Garmyn *et al.*, 1995)	Healthy volunteers (single 120 mg dose, or daily 90 mg β-carotene for 23 days)	No significant photoprotection, clinically (MED) or histologically, of β-carotene against SSR
(Biesalski *et al.*, 1996)	Healthy young females (30 mg β-carotene/day for 10 weeks)	Slight but significant protection against solar radiation-induced skin inflammation with β-carotene
(Boffa *et al.*, 1996)	12 EPP patients (oral ascorbate 1g/daily for 4 weeks)	Slight but non-significant photoprotection (sunlight tolerance)
(Eberlein-König *et al.*, 1998)	10 subjects (either 2 g ascorbic acid combined with 1000 IU of α-tocopherol or placebo per day, for 8 days)	Slight but significant protection ($p < 0.01$) by ascorbic acid and α-tocopherol in combination against UVA/UVB-induced erythema
(Fuchs and Kern, 1998)	40 healthy volunteers (20 – 47 years of age; ascorbic acid and/or α-tocopherol for 50 days; varying dosages)	Significant protection from solar-simulated light-induced erythema of combined ascorbic acid and α-tocopherol ($p < 0.004$)

‡ Photoprotection is variously measured as reduced sunburn cell formation, reduced erythema, reduction of non-melanoma skin cancer.
Abbreviations: EPP, erythropoietic protoporphyria; MED, minimal erythemal dose; NMSC, non-melanoma skin cancer; SSR, solar-simulated radiation

1.12 Aims of the thesis

The relative importance of TR and the GPXs has not been previously studied regarding their role in antioxidant defence of the endothelium and the skin. Therefore this thesis examines the participation of these selenoproteins in the protection against physiological- and non-physiologically mediated oxidative stress in *in vitro* models of the endothelium and the skin. The modification of selenoprotein expression through Se supply and through selenoprotein inhibitors is also studied.

Previous studies demonstrate that Se supplementation, through the modulation of selenoprotein expression, causes BAEC to be resistant to the cytotoxicity of ROS-mediated damage (Geiger *et al.*, 1993; Ochi *et al.*, 1992; Thomas *et al.*, 1993). These investigations attribute the protection observed to modification of GPX expression. Other selenoenzymes such as TR were not considered. Studies have also shown skin cells, primary keratinocytes, HaCaT cells, and fibroblasts, more resistant to UV-induced cytotoxic damage when pre-treated with Se (Emonet *et al.*, 1997; Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b; Stewart *et al.*, 1996). Although other studies in skin cells have demonstrated the modulation of selenoprotein expression by Se supplementation, the protective effect of Se has not been correlated to the expression of the individual selenoproteins.

Thus, the experiments in this thesis were constructed to answer the following questions:

- What effect does Se deficiency have on the ability of cells in culture to withstand oxidative stress?
- Can Se supplementation, in the form of sodium selenite, protect EAhy926 cells from cytotoxicity resulting from exposure to oxidised lipids?
- Can sodium selenite supplementation protect HaCaT cells from cytotoxicity mediated by menadione or irradiation with UVB?
- If sodium selenite supplementation does provide protection, do the concentrations that provide protection maximally upregulate TR, cyGPX and PHGPX in these cell types?
- Does sodium selenite directly detoxify t-BuOOH or menadione, or is the protection through modification of selenoenzyme expression?
- Does gold thioglucose-mediated inhibition of selenoenzyme activity render the cells more susceptible to oxidative stress? Is the magnitude of susceptibility equal for different oxidative stressors? Are the activities of the GPXs and TR equally modulated by gold thioglucose treatment?
- Do any other components of the cell culture systems influence the level of cytotoxicity?
- Are the EAhy926 and HaCaT cell lines suitable models in which to investigate the role of Se and selenoproteins in human endothelial cells and keratinocytes (respectively)?
- Do TR inhibitors quoted in the literature modulate TR activity equally in the DTNB and insulin reduction assay systems for TR activity?

CHAPTER TWO
MATERIALS AND GENERAL METHODS

2.1 Materials: Chemical Suppliers

Cell Culture Reagents and Equipment

Amphotericin B solution; Dulbecco's modified Eagle's medium (DMEM) (25 mM HEPES) with 4.5 g/L glucose; DMEM with glutamax (4.5 g/L glucose); Earle's balanced salt solution (EBSS); Ham's F-12 medium; Hank's balanced salt solution (HBSS) (Ca^{2+}- and Mg^{2+}-free); hypoxanthine, aminopterin, thymidine (HAT) medium supplement; Keratinocyte-SFM medium; Medium 199 (M199); foetal bovine serum (FBS); penicillin/streptomycin solution; phosphate buffered saline (PBS); soyabean trypsin inhibitor; trypsin-EDTA were supplied by Invitrogen Ltd, Paisley, UK.

Bovine aortic endothelial cells (BAEC); endothelial growth medium kit (EGM); endothelial growth medium-2 bulletkit (EGM-2); endothelial growth medium-2 supplements (i.e. HEPES buffered saline solution, trypsin/EDTA solution, trypsin neutralising solution); human coronary artery endothelial cells (HCAEC); human umbilical vein endothelial cells (HUVEC) (certain experiments); were supplied by Biowhittaker UK Ltd., Wokingham, Berkshire, UK.

Tissue culture plastics were supplied by Iwaki, Japan, and Bibby Sterilin, Stone, Staffordshire, UK.

Radioisotopes

Bolton & Hunter reagent for protein iodination (18.5 MBq, 500 μCi) and Iodine-125 (37 MBq, 1 mCi, specific radioactivity 16 MBq/ nmol) were supplied by Amersham International plc, Buckinghamshire, UK.

[^{75}Se] selenite (specific activity, 16 MBq/ nmol) was supplied by Reactor Center, Columbia, MO, USA.

General materials

Acetic acid; Aquamount; copper (II) sulphate; ethanol; ethylenediaminetetraacetic acid (EDTA); hydrochloric acid; methanol; microcrystalline cellulose; orthophosphoric acid; nitric acid; perchloric acid were supplied by MERCK, Leicester, UK.

Acrylamide solution; 2′5′-ADP-agarose columns; ammonium persulphate; anthralin (dithranol); aurothioglucose (gold thioglucose); azelaic acid; bovine serum albumin powder (BSA); Brij solution; butylated hydroxytoluene (BHT); Coomassie brilliant blue (R-250 and G-

250); cytoplasmic glutathione peroxidase purified from human erythrocytes; DEAE Sepharose CL-6B anion exchange column; 5,5′-dithiobis(2-nitrobenzoate) (DTNB); dithiothreitol (DTT); N-ethyl maleimide (NEM); insulin; G-100 gel filtration column; glutathione; glutathione reductase; lactate dehydrogenase (LDH) kit (Sigma Diagnostics); lauryl sulfate (sodium dodecylsulfate; SDS); menadione sodium bisulphite (2-methyl-1,4-napthoquinone); NADPH; p-chloromercuri-benzoic acid (PCMB); phorbol-12-myristate 13-acetate (PMA); radiographic film Kodak X-OMAT XAR-5; reactive blue affinity column; 13-cis retinoic acid; sulphosalicylic acid; sodium azide; sodium selenite; thioredoxin; ,N,N',N'-tetramethylethylene diamine (TEMED); trypan blue solution were supplied by Sigma Aldrich Company Ltd, Poole, Dorset, UK.

Lipoprotein Electrophoresis kit was supplied by Beckman Coulter UK Ltd, Buckinghamshire, UK.

Donkey anti-rabbit serum; normal rabbit serum; normal swine serum were supplied by the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK.

Centricon-10 concentrator tubes; polyethersulfone filters; Amicon 52 filtration units were supplied by Amicon Millipore, Bedford, MA, USA.

Avidin-Biotin Complex Alkaline Phospahatase was supplied by Dako Ltd, Buckinghamshire, UK.

Nitro-Blue tetrazolium chloride; BCIP (5-Bromo-4-chloro-3-indolyl phosphate); 'Complete' Protease Inhibitor; Precinorm®U Universal control serum were supplied by Boehringer Mannheim.

Sephadex G25M columns (PD-10) were supplied by Amersham Pharmacia Bio-Technics, Uppsala, Sweden.

2.2 SOURCES OF NON-COMMERCIAL MATERIAL

2.2.1 Cell lines

2.2.1.1 EAhy926 Endothelial Cell Line

The human endothelial cell line, EAhy926 was a kind donation from Professor Cora-Jean Edgell, the University of North Carolina, North Carolina, USA.

2.2.1.2 Human Keratinocyte HaCaT Cell Line

Cultures of the spontaneously transformed human keratinocyte cell line HaCaT were a kind gift from Professor N E Fusenig, German Cancer Research Centre, Heidelberg, Germany.

2.2.2 Antibodies

Antisera to both rat liver and human placental thioredoxin reductase (TR) were raised in rabbits to cytosolic proteins purified to homogeneity. Rat TR antisera was supplied by Professor John Arthur, Rowett Research Institute, Bucksburn, Aberdeen, UK. Dr Forbes Howie of this department supplied the antisera to human TR.

Antisera to rat PHGPX were raised in rabbits against PHGPX purified from rat testis. This antisera was also kindly donated by Professor John Arthur and Mr Fergus Nicol of the Rowett Research Institute.

2.2.3 Lipoprotein

Normal and oxidised low density lipoprotein was prepared by Mrs Margaret Millar and Professor Rudolph Riemersma of the Cardiovascular Research Department, University of Edinburgh. Donations of plasma were obtained from the National Scottish Blood Transfusion Service, Lauriston Place, Edinburgh.

2.2.4 Human tissue samples

Human placentas, for cytosol preparation, and umbilical cords, for preparation of HUVEC, were obtained from the Simpson Maternity Pavilion, Royal Infirmary of Edinburgh.

Neonatal foreskins, for preparation of primary keratinocytes, were obtained from the Sick Kids Hospital, Edinburgh.

Human foetal and neonatal liver cytosols, for selenoenzyme activity measurements, were obtained from Professor R. Hume of Ninewells Hospital and Medical School, Dundee. The collection of these tissues was approved by the Paediatric-Reproductive Medicine Ethics of Medical Research Sub-Committee of Lothian Health Board and the Ethics Committee of Tayside Health Board.

2.3 GENERAL METHODS

2.3.1 Introduction

The experimental methods and protocols described below are those used throughout this thesis. They include primary cell culture, maintenance of cell lines, selenoprotein determination, cell viability measurements, and selenoprotein expression and activity measurements. Where the methods differ from those detailed here, the modifications are described in the respective chapters.

2.3.2 Cell Culture Conditions for Cell Lines

a) Maintenance of EAhy926 Cell Line

EAhy926 cells were maintained, unless otherwise stated, in high glucose (4.5 g/L) DMEM (containing 25 mM HEPES) supplemented with 10 % FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, 0.8 mM thymidine, in a humidified atmosphere of 5 % CO_2, 95 % air at 37°C. The cells were passaged weekly using 0.25 % trypsin- 0.02 % EDTA solution.

EAhy926 cells display the characteristic morphology of endothelial cells in culture, distinguished by an appearance (via light microscopy) of non-overlapping large polygonal cells, which after 3-7 days in culture became a confluent single monolayer of contact-inhibited cells with a cobblestone appearance. The EAhy926 cell line has previously been shown to stain positive for von Willebrand Factor (former designation Factor VIII-related antigen, vWF) (Edgell *et al.*, 1983). The presence of the glycoprotein vWF was also confirmed by this laboratory (figure 2.01).

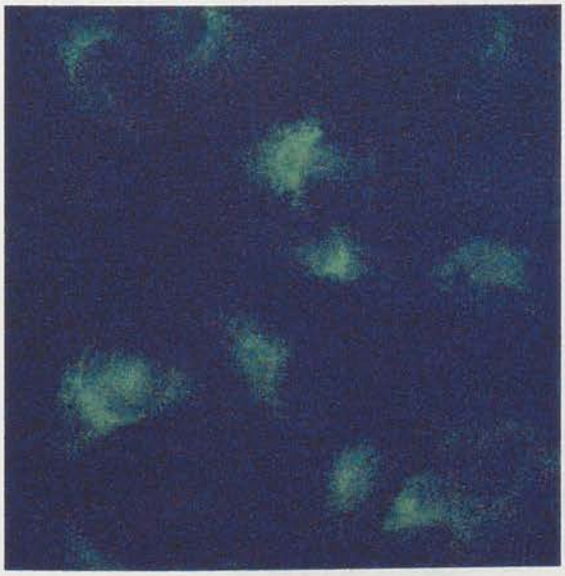


Figure 2.01 Immunofluorescence of the glycoprotein von Willebrand Factor (vWF) in a non-confluent monolayer of EAhy926 cells. x 400 magnification.

b) Maintenance of HaCaT Cell Line

The cell line was maintained, unless otherwise stated, in high glucose (4.5 g/L) DMEM supplemented with 5 % FBS, in a humidified atmosphere of 5 % CO_2, 95 % air at 37°C. The cells were passaged weekly using 0.25 % trypsin-0.02 % EDTA solution.

HaCaT cells in culture display the characteristic morphology of ovoid keratinocytes under the light microscope. HaCaT cells have been shown to behave phenotypically like normal keratinocytes in terms of growth and differentiation, maintaining full epidermal differentiation capacity (Boukamp *et al.*, 1988). This cell line is a spontaneously transformed human epithelial cell line from adult skin, which possesses an enzymatic pattern similar to normal, non-transformed human keratinocytes.

2.3.3 Isolation and/or maintenance of primary cells

a) Isolation and culture of human umbilical vein endothelial cells

Human umbilical cords (>100 mm in length) were obtained at normal deliveries or Caesarean section from non-smoking females. Immediately after delivery the cords were placed into sterile Earle's Balanced Salt Solution (EBSS) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml) at 4°C. Endothelial cells were isolated within 20 hr of delivery using a method adapted from that described previously by Jaffe *et al.* (Jaffe *et al.*, 1973). The vein of the umbilical cord was located and cannulated with a Venflon (gauge 17/ 45 mm), which was then clamped into place. The vein was flushed through with 100 ml of EBSS (pre-warmed to 37°C) to remove any blood clots and the outside wiped using sterile gauze. One end of the cord was clamped shut, and the opposite end infused with 0.07 % collagenase (type IV) in EBSS (5 -15 ml). The cord was then incubated at 37°C in a humidified atmosphere of 5 % CO_2, 95 % air.

After 10 min the cord was removed and massaged gently. The contents of the cord were flushed out with 30 ml of HBSS ($Ca^{2+}$and Mg^{2+}- free). The resulting cell suspension was collected and centrifuged at 450 *x g* for 10 min and the cell pellet washed once with EGM-2 containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). The cells were resuspended in 15 ml EGM-2 and seeded into one 75 cm^2 flask. This flask was then incubated at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air.

After approximately 5 hr the HUVEC were washed with 2 x 10 ml EGM-2 to remove any blood, contaminant cells and cell debris. The medium was then changed and replaced with a further 15 ml of EGM-2 on alternate days.

The cells reached confluence within 3-7 days. When the cells were approximately 90 % confluent the HUVEC were subcultured as required. To subculture the HUVEC the overlying medium was aspirated from the flask and the flask rinsed with approximately 9 ml HBSS. The HBSS was then aspirated from the flask and replaced with 9 ml trypsin (0.025 %) / EDTA (0.01 %) solution. The flask was then placed in the incubator at 37°C for approximately 1.5 min. Cell detachment was checked by light microscope, and if necessary the flask was given a rap to detach any cells remaining attached to the surface of the flask. The trypsin/ EDTA solution was then neutralised with a trypsin-neutralising solution and the cell suspension transferred to a centrifuge tube. The cells were pelleted by centrifugation at 450 *x g* for 10 min. The supernatant was aspirated and the cells gently resuspended in 5 ml EGM-2. The number of cells were counted using a haemocytometer, and were seeded at a density of approximately 3000 cells /cm^2 in a 75 cm^2 flask.

b) Maintenance of human coronary arterial endothelial cells

Human coronary arterial endothelial cells (HCAEC) were maintained in endothelial growth medium-2 bulletkit (EGM-2) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air. Passaging of the cells took place every 7-9 days as described in section 2.3.3.

HCAEC were purchased from Biowhittaker UK Ltd. The certificate of analysis supplied with the cells stated that the HCAEC tested positive for the presence of vWF and acetylated LDL (an alternative method for the specific identification of endothelial cells in culture) (Voyta *et al.*, 1984). HCAEC also displayed the characteristic morphology of endothelial cells as described above and cultures where non-endothelial contaminants were observed were discarded.

c) Maintenance of bovine aortic endothelial cells

Bovine aortic endothelial cells (BAEC) were maintained in endothelial growth medium bulletkit (EGM) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2, 95 % air. The cells were passaged every 7-9 days as described in section 2.3.3.

BAEC were purchased from Biowhittaker UK Ltd. The certificate of analysis supplied with the cells stated that the BAEC tested positive for acetylated LDL. In culture BAEC displayed the characteristic morphology of endothelial cells as described above and cultures where non-endothelial contaminants were observed were discarded.

d) Isolation and culture of epidermal keratinocytes from neonatal foreskins

At circumcision, neonatal foreskins were placed into DMEM. The tissue was then transported on ice, and kept at between 2 and 8°C until use. The foreskins were cut into 3 or 4 pieces of equal size, and then rinsed in sterile petri dishes in 3 x 25 ml PBS (Ca^{2+} -and Mg^{2+}-free) containing antibiotics, fungizone (25 μg/ml) and penicillin (1000 U/ml) / streptomycin (40 μg/ml). The pieces of tissue were returned to a petri dish containing PBS where fat, blood vessels and as much dermis as possible were removed. The pieces of tissue were then placed into a 1 % dispase solution (10 ml) overnight, at 4°C.

Following incubation in dispase, the tissue was removed into a petri dish containing PBS. The epidermal layer was carefully lifted from the dermis using forceps, cut into smaller pieces, and placed into a centrifuge tube containing 50 ml trypsin-EDTA (0.025 %/0.01 % EDTA) solution. The epidermal tissue was incubated with the trypsin-EDTA at 37°C for 10 to 15 min, with occasional vortexing. Once the incubation time had finished, the action of the trypsin was stopped by the addition of 5 % foetal calf serum (v/v). The cell suspension was passed through a cell strainer (70 μm pore size) into a universal tube.

The cells were centrifuged at 500 *x g* for 10 min at RT to gently pellet the cells. The cell pellet was gently resuspended in 5 ml DMEM and placed in a 25 cm^2 tissue culture flask coated with fibronectin. After 1 to 2 days, the DMEM was replaced with Keratinocyte-SFM (GibcoBRL Life Technologies) containing bovine pituitary extract (BPE) (25 μg/ml), recombinant epidermal growth factor (rEGF) (0.1-0.2 ng/ml), fungizone (25 μg/ml) and penicillin (100 U/ml) / streptomycin (100 μg/ml). The cells were maintained in a humidified atmosphere of 5 % CO_2, 95 % air at 37°C.

Upon reaching 60 - 75 % confluency, the keratinocytes were passaged. Since primary keratinocytes show variability donor to donor variability in their growth characteristics, the flasks of cells may take between 10 and 20 days to reach the required 60 - 75 % confluency following the isolation. To subculture the keratinocytes, the overlying medium was aspirated from the flask and the flask washed with approximately 10 ml PBS (Ca^{2+} -and Mg^{2+}-free). The PBS was then aspirated from the flask and replaced with 2 ml trypsin (0.025 %/EDTA 0.01 %) solution. The flask was then placed in the incubator at 37°C for 5 - 10 min. Cell detachment was assessed under by light microscope. When approximately 90 % of the cells had detached, the trypsin/ EDTA solution was neutralised with 10 ml soyabean trypsin inhibitor, and the cell suspension transferred to a centrifuge tube. The cells were pelleted by centrifugation at 500 *x g* for 10 min at RT. The supernatant was then aspirated and the cells gently resuspended in 5 ml Keratinocyte-SFM. The number of cells were counted using a

haemocytometer and seeded at a density of approximately 1 – 3 x 10^6 cells per 75 cm^2 flask in 10 ml Keratinocyte-SFM. Cells were used between passage numbers 2 and 5.

2.3.4 Preparation of Human Placental Cytosol

Normal 40-wk full-term placentas obtained within 2 hr of delivery were used, and all procedures were performed at 4°C or on ice. The placenta (~550 g) was cut into pieces 4 cm^3. Following removal of the umbilical cord and amniotic membrane, the pieces of placenta were washed with 2 L of 50 mmol Tris/HCl, pH 7.4, at RT, containing 1 mmol EDTA, 0.5 mmol DTT (buffer A), and centrifuged at 30,000 *x g* for 1 hr.

2.3.5 Assays for Thioredoxin Reductase (TR) Activity

2.3.5.1 DTNB Assay

a) Sample Preparation

Cell pellets were stored at - 80°C immediately following harvesting. Prior to assay, samples were removed from the freezer, thawed, resuspended in 200 μl (pellets from 75 cm^2 flasks) or 250 μl (pellets from 225 cm^2 flasks) 0.125 M phosphate buffer containing 1 mM EDTA and peroxide-free and carbonyl-free Triton X-100 (0.1 %) and lysed by sonication (three pulses of 10 sec using a Soniprep 150 Sonicator) on ice. The lysates were centrifuged at 500 *x g*, and kept on ice until assay took place. The lysates were diluted 1 in 5 in assay buffer (10 mM EDTA, 0.2 mg/ml BSA in 100 mM potassium phosphate/50 mM potassium chloride buffer, pH 7.0) prior to assay.

Samples were divided into two portions of 180 μl each. One portion was treated with 20 μl of gold thioglucose (GTG) to give a final concentration of 720 nM whilst the other portion was treated with 20 μl of assay buffer. The GTG was freshly prepared on the day of use using assay buffer as diluent, and incubation of sample with fresh GTG was at RT for approximately 10 minutes. The GTG solution was stored in the dark prior to use.

b) Assay Conditions

This assay is based on the conversion of the acceptor substrate DTNB to a yellow chromophore with an absorption max at 412 nm, according to the equation:

DTNB (5, 5´- dithiobis 2-nitrobenzoate) + NADPH + $H^+ \rightarrow$ 2 TNB (thiolate thionitrobenzoate) + $NADP^+$

30 μl of sample was pipetted into a cuvette with 30 μl diluent (distilled H_2O) and 190 μl of reagent. All samples were warmed to 37°C prior to being read. The assay reagent comprised 5 mM DTNB, 0.24 mM NADPH, 10 mM EDTA, 0.2 mg/ml BSA in 100 mM potassium phosphate/50 mM potassium chloride buffer, pH 7.0. EDTA is necessary since mammalian TR is inhibited by heavy metal ions. Assay reagent was stored in the dark prior to use. The assay was performed on a Cobas FARA centrifugal analyser (Roche

Diagnostics, Welwyn Garden City, UK) with absorbance measurement at 412 nm over a period of 0.5 to 290.5 seconds. The absorbance was read once every 10 seconds following an initial reading at 0.5 sec, and the rate read by kinetic analysis over the linear portion of the curve (100.5 to 250.5 seconds). A GTG blank was run in parallel with the samples. The TR activity was calculated as the difference of the reaction rate in the presence and absence of GTG. Results were corrected for protein measured by the Bradford method (section 2.3.9) using BSA as standard.

The extinction coefficient of TNB at 412 nm is 13.6 mM^{-1} cm^{-1} (Holmgren, 1977). TR activity is expressed in units per gram protein (U/g). One unit of mammalian TR activity is defined as 1 μmol TNB formed per minute (Holmgren and Björnstedt, 1995).

c) *Quality control*

Four internal controls were prepared by diluting a pooled stock solution of human placental cytosol with FBS at two-fold, four-fold, sixteen-fold and thirty two-fold dilutions. The controls were constructed to cover the range of activities given by the different cell types in the assay, as assessed by historical data (~ 0.25 to 5.0 U/g protein). The controls were dispensed into 180 μl aliquots and frozen at - 80°C. Good linearity was demonstrated for dilutions of human placental cytosol in the assay.

d) *Optimisation of gold thioglucose concentration in the DTNB assay*

In the original DTNB assay method described by Hill *et al.* (Hill *et al.*, 1997), a concentration of 20 μM GTG was used to selectively inhibit TR activity to increase the specificity of the assay. Sample (TR source) is run in the presence and absence of GTG, with the difference between the two values of DTNB reduction (A_{412}) being the activity due to TR. The concentration of GTG can be tailored to inhibit certain selenoenzymes by virtue of the fact that different selenoenzymes have differing sensitivities to inhibition by GTG (table 2.01). The GPXs are relatively resistant to inhibition whilst TR is very sensitive, having an IC_{50} ~ 1000-fold lower than that of the GPXs (Gromer *et al.*, 1998). Dose-response studies with GTG and human placental cytosol suggested that a final concentration of 727 nM GTG was optimal for maximal TR inhibition (figure 2.02). The apparent disparity between TR activity of the control cytosol (minus GTG) and the cytosol containing very low GTG concentrations may be due to a dilution effect in the cytosol containing GTG, which was not accounted for in the control cytosol.

The final GTG concentration required to inhibit cyGPX activity in human placental cytosol was 1000 μM, which inhibited cyGPX activity by 90 % in human placental cytosol (section 2.3.7) (figure 2.03).

Our studies revealed that GTG degrades if stored frozen in solution. In all studies GTG solutions were prepared fresh on the day of use, and used immediately. Dithiothreitol (DTT) was observed to give high background values in this assay system, requiring some samples to be treated to remove DTT prior to assay for TR.

Table 2.01 IC_{50} values for the inhibition of human selenoenzymes by the gold compounds aurothioglucose (gold thioglucose) and auranofin (from Gromer *et al.*,1998).

	IC_{50} value	
	Aurothioglucose	Auranofin
Thioredoxin reductase	65 nM	20 nM
Glutathione reductase	> 100 μM	40 μM
Glutathione peroxidase	80 μM	> 100 μM

f) Intra-assay precision data

Precision data for the intra-assay variation from one assay (3 duplicates of each pool were read during one assay run), is shown in table 2.02.

Table 2.02 Intra-assay variation for four QC pools run in one assay for TR activity (CV denotes the Coefficient of variation)

QC pool	n	Mean TR activity (U/g protein)	SD	CV (%)
1	3	4.992	0.936	18.8
2	3	2.652	0.156	5.9
3	3	1.560	0.078	5.0
4	3	0.298	0.037	12.5

g) Inter-assay precision data

Inter-assay precision data was determined by running the controls in each assay for 11 consecutive assays. The results are shown in table 2.03.

Table 2.03 Inter-assay variation for four QC pools run in eleven consecutive assays for TR activity (CV denotes the Coefficient of variation)

QC pool	n	Mean TR activity (U/g protein)	SD	CV (%)
1	11	5.078	1.042	20.5
2	11	2.338	0.492	21.1
3	11	1.280	0.274	21.4
4	11	0.310	0.053	17.1

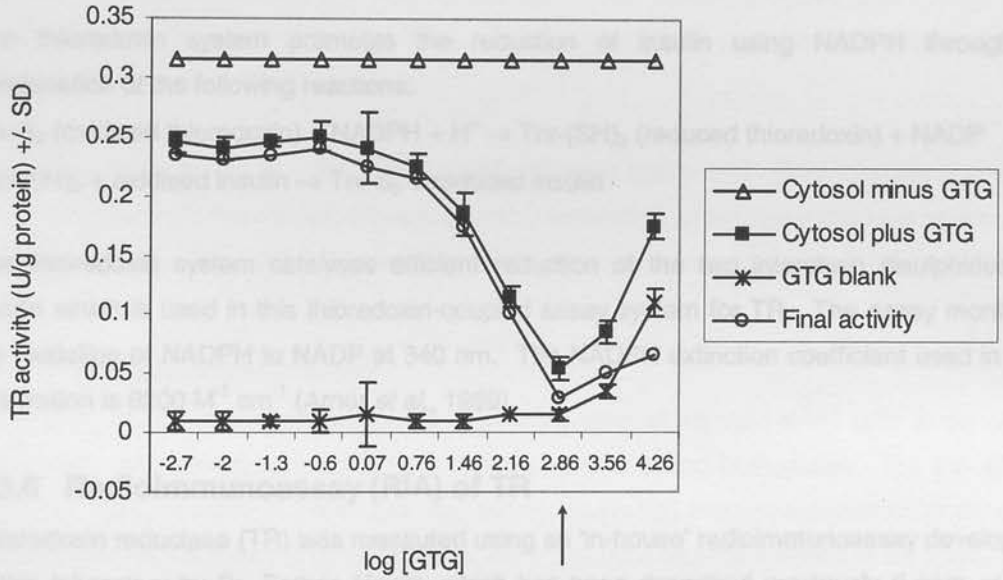


Figure 2.02 The effect of gold thioglucose (GTG) on thioredoxin reductase (TR) activity in human placental cytosol. The GTG was added to the cytosol at final concentrations of 0.00186, 0.0093, 0.047, 0.233, 1.16, 5.8, 29, 145, 727, 3636, and 18,181 nM. The arrow indicates the GTG concentration chosen to give optimal TR inhibition (> 90 %) whilst giving the lowest possible GTG blank rate (727 nM GTG). Results shown are those of the mean of two experiments, where each GTG concentration added to cytosol was read in duplicate. Where the error bars are not visible, they lie within the symbol. The same preparation of human placental cytosol, and same preparation of GTG was used for each of the two experiments.

2.3.5.2 Insulin Assay for TR

a) Sample preparation

Sample preparation was as described in section 2.3.2.1 for the DTNB assay, except that GTG was not added to samples.

b) Assay conditions

20 µl of sample was pipetted into a cuvette with 180 µl of assay reagent. The samples were warmed to 37°C for 120 sec prior to being read. The assay reagent comprised 0.8 mM insulin, 27 mM NADPH, and 0.4 mM oxidised thioredoxin in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA. Assay reagent was stored in the dark prior to use. The assay was performed on a Cobas FARA centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK) with absorbance measurement at 340 nm over the period 0.5 to 250 sec. The absorbance was read once every 5 sec following an initial reading at 0.5 sec, and the rate read by kinetic analysis.

The thioredoxin system promotes the reduction of insulin using NADPH through a combination of the following reactions:

$Trx\text{-}S_2$ (oxidised thioredoxin) + NADPH + $H^+ \rightarrow Trx\text{-}(SH)_2$ (reduced thioredoxin) + NADP

$Trx\text{-}(SH)_2$ + oxidised insulin $\rightarrow Trx\text{-}S_2$ + reduced insulin

The thioredoxin system catalyses efficient reduction of the two interchain disulphides of insulin which is used in this thioredoxin-coupled assay system for TR. The assay monitors the oxidation of NADPH to NADP at 340 nm. The NADPH extinction coefficient used in the calculation is 6200 M^{-1} cm^{-1} (Arnér *et al.*, 1999).

2.3.6 Radioimmunoassay (RIA) of TR

Thioredoxin reductase (TR) was measured using an 'in-house' radioimmunoassay developed in this laboratory by Dr. Forbes Howie, which has been described previously (Lewin *et al.*, 2001; Miller *et al.*, 2001).

a) Preparation of antisera

Antisera were raised in rabbits to an immunogen of human TR purified from placenta. The secondary antibody was prepared by adding 25 ml of donkey anti-rabbit serum to 1.5 ml normal rabbit serum and mixing overnight at RT to precipitate out the immunoglobulins. Following centrifugation (230 *x g*, 5 min), the supernatant was discarded and the precipitate washed four times with 0.05 M phosphate buffer (pH 7.4) containing 0.1 % BSA and 0.02 % sodium azide. After the final wash the supernatant was removed and the pellet was resuspended in the same 0.05 M phosphate buffer as described above, to give a final volume of 100 ml.

b) Preparation of the assay diluent

The assay was performed using a diluent of 25 mmol/L potassium phosphate buffer, pH 7.5, containing BSA 1 g/L, NaN_3 0.2 g/L, and 10 mmol/L dithiothreitol. This diluent was used for both the antibody and the tracer.

c) Preparation of ^{125}I-TR tracer, standards, controls and samples

Purified human placental TR1 was iodinated by Dr Forbes Howie using the Bolton-Hunter iodination procedure (Bolton and Hunter, 1973). The ^{125}I-TR tracer was immediately diluted 1:2 with FBS and stored at -20°C for up to three months. For use in the radioimmunoassay the ^{125}I-TR tracer was diluted with assay diluent such that approximately 10,000 cpm was added to each tube (mass approximately 50-100 pg TR/ tube).

Standards were prepared by diluting a stock solution of purified placental human TR1 (1 mg TR/L) with FBS to give the following concentrations: 0.5, 1, 2, 5, 10, 25, 50 μg TR/L. Two controls were made by diluting a stock solution of human placental cytosol with FBS to give values of approximately 5 and 25 μg TR/ L. Both standards and controls were dispensed into aliquots and frozen at - 80°C.

Cell pellets were stored at - 80°C immediately following harvesting. Prior to assay, samples were removed from the freezer, thawed, lysed by sonication, centrifuged and kept on ice until assay took place. Each sample was diluted with radioimmunoassay buffer between 1:50 and 1:200.

d) TR radioimmunoassay

Antibodies, tracer, standards, controls and samples were all warmed to RT prior to set up of the assay. All samples, standards and controls were assayed in duplicate. For the assay 100 μl of ^{125}I-TR tracer (~15,000 cpm) was pipetted with 100 μl standard, control or sample. Primary anti-TR1 antibody (100 μl; final dilution 1 : 30,000) was then added to all tubes, with the exception of the total counts, which were vortexed and incubated at 4°C overnight.

The following day, pre-precipitated second antibody (donkey anti-rabbit serum) prepared as described above (section 2.3.6 a) was added to each tube (except the total counts), vortexed, and incubated at RT with shaking for 1 hour. Following this, 1.5 ml wash solution (0.05 % v/v Brij 35 and 0.001 % w/v microcrystalline cellulose) was added (except to the total counts tubes) and the tubes centrifuged at 3000 *x g* for 30 min at 4°C. The supernatants were then removed by decanting and the radioactivity in the pellet counted on a 1261 MULTIGAMMA Gamma Counter (Wallac, Gaithersburg, MD, USA). The standard curve was plotted and results interpolated using a multicalc data processessing package (Wallac, Gaithersburg, MD, USA).

Results were corrected for protein measured by the Bradford method (section 2.3.9). The intra-assay precision of the TR radioimmunoassay was < 10 % coefficient variation over the range of concentrations measured. Controls were run as 9 replicates within a single assay to determine intra-assay precision. To determine the inter-assay precision, controls were included at the beginning and end of each assay.

e) Intra-assay precision data

The intra-assay variation was calculated from one assay (in which 9 duplicates of each pool (5 and 25 μg TR/ L) were read from a single standard curve, is shown in table 2.04.

Table 2.04 Intra-assay variation for two pools run in one assay for TR concentration (CV denotes the Coefficient of variation)

pools	n	Mean TR concentration (μg/protein)	SD	CV (%)
5 μg TR/ L	9	5.062	0.733	14.473
25 μg TR/ L	9	24.391	0.963	3.949

f) Inter-assay precision data

The inter-assay precision data was determined by running the controls in each assay for 12 consecutive assays. The results are shown in table 2.05.

Table 2.05 Inter-assay variation for two pools run in twelve consecutive assays for TR concentration (CV denotes the Coefficient of variation)

pools	n	Mean TR concentration (μg/g protein)	SD	CV (%)
5 μg TR/ L	12	5.569	0.446	8.009
25 μg TR/ L	12	25.151	2.811	11.176

g) Detection range of TR radioimmunoassay

A precision profile was constructed to determine the functional sensitivity of the assay, calculated from 9 consecutive assays. The minimum detection limit of the TR RIA was calculated as 1.50 μg TR/L which represented the TR concentration which had a CV of 22.5 %. The working range of this assay was 3.50 - 50.0 μg TR/L and was defined as the concentration range which had a CV of less than 10 %.

2.3.7 Cytosolic glutathione peroxidase (cyGPX) assay

Cell pellets were stored at - 80°C immediately following harvesting. Samples were then transported on dry ice to the Rowett Research Institute, Aberdeen, UK where cytosolic glutathione peroxidase (cyGPX) activities were measured by Miss Karen Pickering or Mr Fergus Nicol of the Rowett Research Institute using a method previously described by Beckett *et al.* (Beckett *et al.*, 1990). Some cyGPX assays were carried out by the author at the Rowett Research Institute.

cyGPX activity in human placental cytosols was measured in-house using an assay method adapted for use on the Cobas Fara centrifugal analyser.

cyGPX activity was determined by following the rate at which $NADPH_2$ is converted to $NADP^+$ measured as a change in absorbance at 340 nm in the presence of the substrate hydrogen peroxide (detailed in equation below), using a Unicam UV/Vis spectrometer (UV4) linked to a computer installed with 'Vision' software in the case of cell lysates, or using a Cobas Fara centrifugal analyser.

The reaction catalysed by cyGPX utilised for its assay is as follows:

2GSH (reduced glutathione) + H_2O_2 (hydrogen peroxide) $\rightarrow$ GSSG (oxidised glutathione) + $2H_2O$

Reduced glutathione, hydrogen peroxide, glutathione reductase and $NADPH_2$ are added in excess such that the rate limiting step is glutathione peroxidase. The GPX activity is directly related to the rate of change of $NADPH_2$ to $NADP^+$ which is followed at 340 nm.

A unit of cyGPX is defined as that which oxidises 1 μmole of NADPH per min. The molar extinction coefficient of NADPH is 6200 M^{-1} cm^{-1}. The blank rate was subtracted from the rate obtained after substrate addition. The conversion factor for the assay when a 20 μl sample was used was 8.0385.

a) Preparation of samples and standards

The homogenisation buffer was prepared as follows. 50 μl aliquots of peroxide-free, carbonyl-free Triton X-100 were stored under argon in HPLC vials. 50 μl of Triton X-100 was removed from a vial with a syringe into an Eppendorf tube, and kept on ice. 50 ml of buffer (0.125M KPO_4; 1 mM EDTA; pH 7.4) was placed in a beaker and kept on ice. The Triton X-100, to solubilise cell membranes, was added to the buffer, which was thoroughly stirred.

Cell pellets were thawed and resuspended in 200 μl (pellets from 75 cm^2 flasks) or 250 μl (pellets from 225 cm^2 flasks) 0.125 M phosphate buffer containing 1 mM EDTA and peroxide-free and carbonyl-free Triton X-100 (0.1 %) as described above. The cell pellets

were lysed by sonication (three pulses of 10 sec) on ice. Purified cyGPX was used as a positive control.

b) Preparation of the reaction mix

A reaction mix was made up of the following: 5 mg $NADPH_2$, 46 mg reduced glutathione, 3 ml distilled H_2O, 24 ml PBS (pH 7.6), 1 ml sodium azide (112.5 mM), 20 U glutathione reductase and 0.1 % peroxide- and carbonyl-free Triton X-100. The reaction mix was placed in a water bath at 37°C.

To measure cyGPX activity using a Unicam UV/Vis spectrometer, 990 μl reaction mix was added to 10 μl sample in a 1 ml glass cuvette. The blank rate was followed for 5 cycles at 340 nm for approximately 3 min. To start the reaction 20 μl of the substrate hydrogen peroxide solution (2.2 mM) was added, and the subsequent reaction rate was followed for a further five cycles at 340 nm.

To measure cyGPX activity in human placental cytosol using a Cobas Fara centrifugal analyser, 15 μl sample was automatically pipetted along with 30 μl diluent (distilled H_2O) and 180 μl reaction mix into a cuvette. The blank rate with H_2O in place of the substrate was also measured. The reaction was started by addition of 5 μl of the hydrogen peroxide substrate solution, and the subsequent reaction rate was followed for 100.5 sec at 340 nm. The first reading was taken at 0.5 sec, with each subsequent reading at 5 sec intervals.

The concentration of gold thioglucose (GTG) required to inhibit cyGPX activity in human placental and human liver cytosols was investigated to compare to results for inhibition of TR activity in human placental cytosol. Inhibition of cyGPX activity in human placental and liver cytosols by GTG concentrations of 1, 10 and 100 μM GTG did not produce any loss of cyGPX activity in human placental or liver cytosol (figure 2.03 a). The IC_{50} of GTG for cyGPX activity in human placental cytosol and liver cytosol was ~ 150 μM and 300 μM, respectively. In comparison, the IC_{50} of GTG for TR activity in human placental cytosol was ~ 87 nM (figure 2.02). These data are comparable to those of Gromer *et al.* (Gromer *et al.*,1998) (table 2.01).

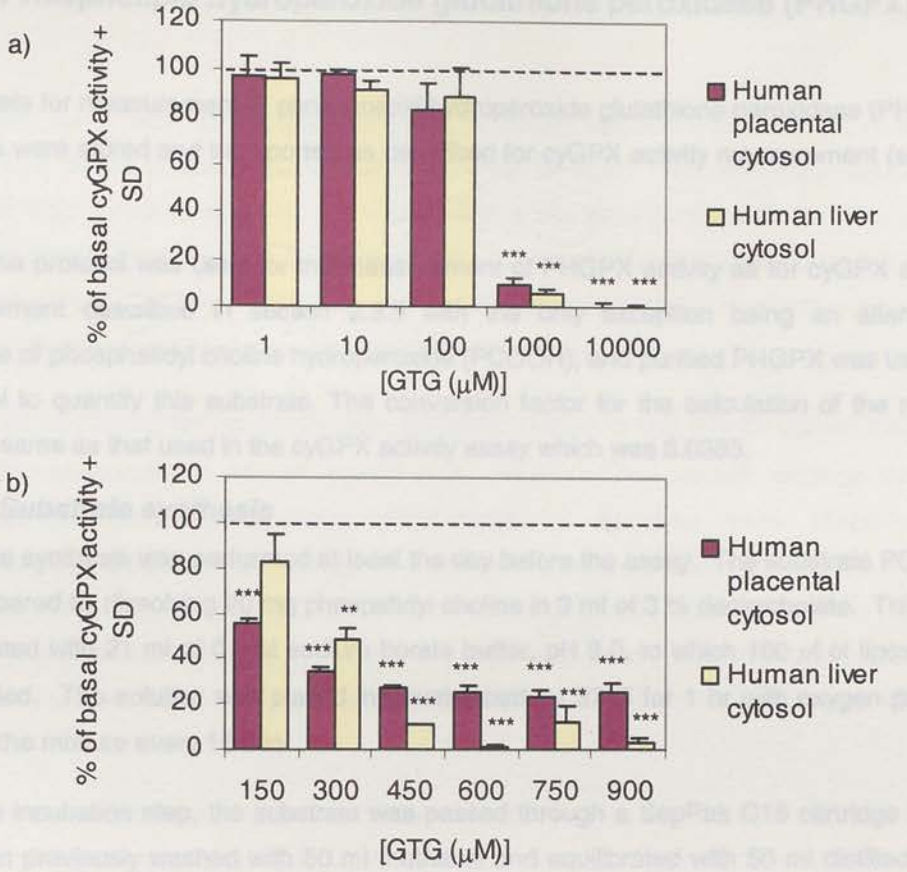


Figure 2.03 Inhibition of cytoplasmic glutathione peroxidase (cyGPX) activity in human placental and liver cytosols by gold thioglucose (GTG). GTG was added at a final concentration of (a) 1 to 10,000 μM or (b) 150 to 900 μM. Results shown are those of the mean of duplicate samples + SD. The basal level of activity in control cytosols is indicated by the dashed line. $p < 0.01$**, $p < 0.001$*** cf. level of activity in control cytosol.

2.3.8 Phospholipid hydroperoxide glutathione peroxidase (PHGPX) assay

Cell pellets for measurement of phospholipid hydroperoxide glutathione peroxidase (PHGPX) activities were stored and transported as described for cyGPX activity measurement (section 2.3.5).

The same protocol was used for the measurement of PHGPX activity as for cyGPX activity measurement described in section 2.3.5 with the only exception being an alternative substrate of phosphatidyl choline hydroperoxide (PCOOH), and purified PHGPX was used as a control to quantify this substrate. The conversion factor for the calculation of the results was the same as that used in the cyGPX activity assay which was 8.0385.

a) Substrate synthesis

Substrate synthesis was performed at least the day before the assay. The substrate PCOOH was prepared by dissolving 20 mg phospatidyl choline in 3 ml of 3 % deoxycholate. This was then diluted with 21 ml of 0.2 M sodium borate buffer, pH 9.0, to which 100 μl of lipoxidase was added. The solution was placed in a water bath at 37°C for 1 hr with oxygen passed through the mixture every 15 min.

After the incubation step, the substrate was passed through a SepPak C18 cartridge which had been previously washed with 50 ml methanol and equilibrated with 50 ml distilled H_2O. The column was than washed with 50 ml distilled H_2O and all the air flushed out all with a 20 ml syringe. The PCOOH substrate was eluted into a clean tube with 2 ml methanol. The substrate could be stored at -40°C for up to 2 months. A unit of PHGPX is defined as that which oxidises 1 μmole of NADPH per min.

2.3.9 Bradford Assay

Protein measurements were carried out utilising the dye-binding assay of Bradford (1976) (Bradford, 1976) adapted for use on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK).

The Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95 % ethanol. To this solution was added 100 ml 85 % (w/v) phosphoric acid, and the mixture stirred for 30 min. The resulting solution was diluted with distilled H_2O to a final volume of 1000 ml, filtered through Whatman grade 1 filter paper and stored at RT in a closed bottle.

Bradford reagent (256 μl) was added to each cuvette, which was incubated for 100 sec at 37°C prior to sample addition, with an initial absorbance reading (595 nm) taken at 95 sec.

Following the addition of 25 μl of sample plus 50 μl distilled H_2O (diluent) to the cuvettes, a further incubation took place for 180 sec at 37°C. A final absorbance was then read at 595 nm.

A standard curve was constructed using bovine serum albumin (BSA) as standard, and distilled H_2O as the diluent, covering the range 0 to 100 mg/L. The difference between the final and initial absorbances was calculated, and a standard curve plotted. The protein concentrations of samples were interpolated from the standard curve. Samples were diluted with distilled H_2O to fall in the middle portion of the standard curve. A quality control (QC) was run with every rotor to assess the reproducibility of the results.

2.3.10 Sodium-dodecyl sulphate- polyacrylamide gel electrophoresis

The [^{75}Se]-selenoproteins in cell lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared for electrophoresis by diluting each sample to a common protein concentration with 60 mM Tris buffer, pH 7.8 at RT, containing 1 mM EDTA and 1 mM dithiothreitol. The diluted samples were further diluted 2:1 (sample: "boiling mix") with "boiling mix" (SDS 35 mM, glycerol 1.4 mM, 2-mercaptoethanol 0.3 mM and bromophenol blue 15 mM) and heat-treated at 90°C for 10 min.

One-dimensional electrophoresis was carried out at RT using a Protean II electrophoresis system (Bio-Rad Laboratories Ltd, Watford, Herts, UK). The discontinuous gel (0.1 cm x 16.5 cm x 18 cm) was cast as two separate gels sandwiched between two glass plates, set in a casting stand. The lower 12 % resolving gel (14 cm long) was made up of 17 ml distilled H_2O; 32 ml acrylamide solution (30 % acrylamide, 0.8 % bis-acrylamide); 30 ml 1M Tris/ HCl, pH 8.85; 0.8 ml 10 % SDS; 0.15 ml N,N,N',N'-tetramethylethylene diamine (TEMED); 0.15 ml 0.1 % ammonium persulphate. The upper stacking gel (approximately 4 cm long) consisted of 32 ml distilled H_2O; 7.2 ml acrylamide; 20 ml 0.375 M Tris/ HCl, pH 6.8; 0.6 ml 10 % SDS; 0.2 ml TEMED; 0.2 ml 0.1 % ammonium persulphate. Once set the gel sandwiches were removed from the casting stand and transferred to the buffer chamber. The upper and lower buffer chambers were filled with SDS-electrophoresis buffer (0.3 % (w/v) Tris; 1.44 % glycine; 0.1 % SDS); low range molecular weight markers (14.4 kDa – 97.4 kDa) and samples were loaded into the wells. The gel was run at 200 volts (V), 35 milliamperes (mA), 50 watts through the stacking gel until the bromophenol blue tracking dye entered the resolving gel, when the voltage and current was increased to 300 V and 50 mA respectively. The power supply was disconnected once the tracking dye had reached the bottom of the resolving gel.

The gels were stained in 0.2 % (w/v) Coomassie Brilliant Blue R in a methanol: acetic acid: distilled H_2O solution (50: 7: 50 ratio) for 30 min, and then destained in two changes of a methanol: acetic acid: distilled H_2O solution (5: 7: 88 ratio) overnight.

The gels were then dried under vacuum in a Bio-Rad gel drier (model 583) at 80°C for 2 hr, sandwiched between two layers of pre-soaked cellophane.

2.3.11 Autoradiography of SDS-PAGE gels

[^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film. The dried gels were placed next to film within an exposure cassette at - 80°C for between 12 hr and 4 days. Films were developed by the Department of Radiology, The Royal Infirmary of Edinburgh. The molecular weights of the standard protein molecular weight markers were plotted against the distance travelled, and a curve fitted using the program Fig P (Fig P Software Incorporation, Durham, NC, USA), which was used to determine the molecular weights of selenoproteins.

2.3.12 Preparation of low-density lipoprotein

Low density lipoprotein (LDL) was isolated from 300-450 ml outdated human citrate plasma (Blood Transfusion Service, Edinburgh) by ultracentrifugation using a Ti 45 rotor in a Beckman instrument (L8.55 ultracentrifuge, Beckman, Glenrothes, UK).

Plasma (45 ml) was overlayered with 15 ml of a buffered saline solution (ρ1.019 g/ml) containing EDTA (10 mg/L) and centrifuged for 18 hr at 186,000 x g_{max} at 4°C. The very low density lipoproteins were discarded. The density of the infranatant was adjusted to 1.063 g/ml by addition of 14.7 ml of buffered saline (ρ 1.182 g/ml), overlayered with 5.3 ml of saline solution (ρ 1.063 g/ml) and re-centrifuged for 18 hr at 186,000 *x g*. The LDL fraction was removed and transferred into a 30 cm long dialysis tube (7.5 mm diameter, Spectra/Por, MW cut-off 300,000; Medicel, London) and dialysed overnight against 5L PBS (pH 7.4, 0.2 g/L chelex resin). The combined dialysates of known protein concentration were divided, one part was kept as native LDL (control experiments) and the other was used to prepare oxidised LDL (oxLDL) as follows. Approximately 25 ml native LDL was oxidised at 37°C using 20 μM $CuCl_2$ (Cu-protein ratio 0.16 μmoles/mg protein), and the formation of conjugated dienes monitored at 234 nm. A Pye Unicam SP8-100 UV Spectrophotometer, equipped with a Techne Circulator C400 set at 37°C, was used to monitor diene formation at 234 nm, using matched quartz cuvettes (1 cm path length). At peak absorbance (usually 60-90 min after initiation), 10 % excess EDTA was added to stop lipid peroxidation and the volume of the lipoprotein fraction was reduced to ~ 5-10 ml using a 30,000 MW cut-off polyethersulfone filter and an Amicon 52 filtration unit (Amicon Millipore, Bedford, MA, USA). Traces of Cu^{2+} were then removed by chromatography over a Sephadex G25M column (PD-10; Amersham Pharmacia Bio-Technics, Uppsala, Sweden) using PBS as the eluent (one pass). The tube containing oxLDL was flushed with a 0.22 μ-filtered stream of argon and

stored at 4°C until required. Native LDL was treated in an identical manner (filtration, chromatography, storage under argon) except the fraction was never exposed to Cu^{2+}.

Both native and oxidised LDL were stored under argon, at 4°C, prior to use. LDL was used for toxicity experiments within 3 weeks of preparation.

2.3.13 Lactate dehydrogenase activity assay for cell damage measurements

Cell damage was assessed as the percentage retention of lactate dehydrogenase (LDH) by the cell layer. Intracellular and extracellular LDH activity, in the cell lysates and culture medium respectively, was determined by following the rate at which NADH is oxidised to NAD^+ measured as a decrease in absorbance at 340 nm in the presence of pyruvate using an LDH kit method (Sigma Diagnostics Ltd, Poole, UK) modified for use on the Cobas Fara centrifugal analyser (Roche Diagnositcs, Welwyn Garden City, U.K.). The rate of decrease in absorbance at 340 nm, measured at 37°C, is directly proportional to LDH activity in the sample. The results were expressed as % LDH activity retained, calculated as follows: intracellular LDH activity/(extracellular LDH activity + intracellular LDH activity) x 100

a) Preparation of reagents

LDH reagent A, when reconstituted, contained NADH (0.194 mM) in phosphate buffer, pH7.5 (54 mM). LDH reagent B, when reconstituted, comprised a solution of pyruvate (16.2 mM) to which non-reactive stabilisers and filler had been added. Both reagents were reconstituted by the addition of deionized H_2O as indicated on the vial. After addition of H_2O, the vial was stoppered and mixed by gentle inversion several times.

b) Sample Preparation

All cells for cell viability studies were plated into 24 well plates at a density of 5×10^5 cells/cm^2 for EAhy926 cells, 2×10^5 cells/cm^2 for HaCaT cells, and 3,000 cells/cm^2 for HUVEC. Immediately after treatment with t-BuOOH, menadione or oxidised LDL the culture medium (1 ml) was removed from the cells and placed into pre-labelled Eppendorfs tubes. Cells were then washed twice with 1 ml phosphate buffered saline (PBS, pH 7.4) and the cells lysed in 0.5 ml of 0.5 % Triton X-100 (in PBS, pH 7.4). After 15 min the cell lysates were collected into pre-labelled Eppendorfs tubes. The wells were washed with a further 0.5 ml PBS and the washings combined with the respective lysates. Cell debris in the culture medium and cell lysates was removed by micro-centrifugation of all samples at 11,000 *x g* for 10 min prior to assay for LDH activity.

c) *LDH activity assay*

Samples (100 μl) were transferred into Cobas cups for LDH activity measurement. The change in absorbance at a wavelength of 340 nm was measured in 10 μl of sample in a final volume of 290 μl (250 μl reagent A and 10 μl reagent B, and 20 μl of distilled H_2O as diluent). The sample, diluent and reagent A were pipetted into the cuvettes and incubated at 37°C for 60 sec before addition of reagent B and diluent. The change in absorbance of the reaction mixture was read at 0.5 sec, and was then read every 5 sec for a total of 20 readings. The LDH activity was expressed as units per litre (U/L) as determined by kinetic analysis.

The LDH activity was also measured in culture media that had not been in contact with cells as a measure of endogenous LDH in the culture medium, as well as Triton X-100 and PBS (lysis conditions). These blank values were subsequently subtracted from each extracellular and intracellular LDH activity. The LDH activity in both the medium and cell lysates was then calculated as described above. Precinorm® U (a commercially produced 'universal control serum'; Boehringer Mannheim) was used for quality control for every run of the assay.

2.3.14 Trypan Blue Assay for cell damage measurements

a) *Sample Preparation*

As detailed in section 2.3.13, all HaCaT cells for viability studies were passaged into 24 well plates. Immediately after the respective treatment period with menadione, or following 48 hr after UVB treatment, the culture medium (1 ml) was removed from the cells and placed into pre-labelled Universal tubes and put aside. The cells were then washed twice with 1 ml EBSS, and all the cells except the first two wells of the 24 well plate received fresh medium. The cells from the first two wells were trypsinised with 0.25 % trypsin-EDTA (0.5 ml). Cell detachment was checked by light microscope, and the trypsin neutralised with FBS-containing medium (0.5 ml). The cell suspension was then added to the medium that had previously been put aside, and centrifuged at 500 *x g* for 10 min. The trypsin-medium was aspirated from the cells, and the cell pellet gently re-suspended in 100 μl EBSS.

b) *Assessment of cell damage*

10μl of the cell suspension was mixed with 10 μl of 0.4 % trypan blue solution, and 10μl of the resultant mixture added to a haemocytometer. The suspension was then left for 1 min to allow the cells to settle; the cells remained uncounted for no longer than 2 min since viable cells begin to take up the trypan blue stain after this time.

One hundred cells were counted, noting the proportion of these cells that had taken up the dye. The number of cells that had taken up the dye gave a measurement of the percentage of damaged/non-viable cells, calculated as follows: (N^o blue cells/Total cells counted) x 100. The scoring of cells was by two independent observers, one reading blind.

For experiments in which cell viability assessments were being compared for both LDH release and trypan blue uptake for the same cells, the culture medium (1 ml) that had been removed from the cells and placed into pre-labelled Universal tubes was centrifuged at 500 *x g* for 10 min. The medium was removed into Eppendorf tubes for LDH analysis (being re-centrifuged at 11,000 *x g* for 10 min prior to LDH assay), while the cell pellet was treated as described above for trypan blue analysis.

2.3.15 Assay of total glutathione

The total glutathione (oxidised plus reduced) concentration of cell lysates was determined using the method of Tietze (Tietze, 1969) adapted for use on the Cobas Fara centrifugal analyser (Roche Diagnositcs, Welwyn Garden City, UK).

The assay reagent was prepared by dissolving 0.007 g NADPH and 0.010 g DTNB in 25 ml assay buffer (150 mM $NAPO_4$; 7.5 mM EDTA, pH 7.5). The start reagent was prepared by diluting glutathione reductase to a final concentration of 10 U/ml with assay buffer (150 mM $NAPO_4$; 7.5 mM EDTA, pH 7.5).

a) Sample preparation

Cell lysates were deproteinised using 3.3 % sulphosalicylic acid. The cells in each well of the 24 well culture plate were washed twice with 1 ml PBS. The 3.3 % sulphosalicylic acid (100 µl/well) was then added to each well, and the plate was placed in a - 80°C freezer for 15 min. The plate was then removed from the freezer to allow to defrost, and the freeze/thaw cycle repeated. The cell lysates were centrifuged at 13,500 *x g* for 5 min prior to assay for total glutathione.

b) Assay conditions

Sample (10 µl), diluent (25 µl H_2O), and assay reagent (150 µl) were pipetted into each cuvette and incubated for 60 sec at 37°C. The start reagent (50 µl) was then added to each cuvette, and reading commenced. The initial absorbance reading (412 nm), was taken at 0.5 sec; after this readings were taken every 5 sec for a total of 50 sec. The rate was then determined by kinetic analysis.

c) Preparation of standard curve

Glutathione (GSH) standards were used at a concentration range of between 0-100 µmol/L. Unless otherwise stated GSH standards were dissolved in 3.3 % sulphosalicylic acid. The change in absorbance reading resulting from the addition of the standard was used to construct a standard curve. Samples with unknown GSH concentrations were then interpolated from this curve.

2.3.16 Measurement of Se content of individual cell culture media

The Se contents of cell culture media were determined by acid digestion followed by fluorimetric analysis (Boyne and Arthur, 1986; Olsen *et al.*, 1975). The method used is based on the formation of piazselenol between Se and 2,3-diaminonaphthalene, followed by extraction with cyclohexane and fluorometry. The samples were read from a standard curve, prepared from 1 mg/ml selenious acid. A dried blood standard was used as a control. These analyses were performed by Mr Fergus Nicol of the Rowett Research Institute, Aberdeen, UK.

2.3.17 Measurement of HDL cholesterol in foetal bovine serum

The foetal bovine serum (FBS) used to supplement cell culture media was measured for its LDL content since several experiments involved adding exogenous oxLDL as a cytotoxic agent. The endogenous LDL in FBS was estimated by measurement of the HDL cholesterol, using an assay system based on formation of HDL-polymer and LDL-polyanion complexes, followed by destruction of HDL particles by detergent, and initiation of an enzyme reaction with HDL-derived cholesterol. Only the HDL in cholesterol is subject to cholesterol measurement. The N-geneous™ HDL Cholesterol Reagent used was supplied by the Genzyme Corporation, Cambridge, Massachusetts, USA.

2.3.18 Electrophoresis of Lipoproteins

Lipoprotein samples were separated by electrophoresis at low voltage using agarose gels according to their net charge at pH 8.6. A Lipoprotein Electrophoresis kit was used (Beckman, CA) in conjunction with a Paragon Electrophoresis System (Beckman Instruments, Palo Alto, USA). Increased electrophoretic mobility was used as a measure of oxidation of the LDL protein moiety.

Lipoprotein samples were loaded onto pre-cast agarose (0.5 %) gels at a volume of 5 μl. The samples were left for 5 min to diffuse into the gel. Excess sample was then removed with a gel blotter, and the gel was placed into a Gel Bridge Assembly. The Gel Bridge Assembly was then placed into a Paragon Electrophoresis cell containing 45 ml B-2 barbital buffer (50 mmol/L 5,5-Diethylbarbituric acid sodium salt, pH 8.6). This was covered with a lid, and the complete Electrophoresis cell inserted into a Paragon power supply unit. The gel was electrophoresed at 100 V for 30 min. Upon completion of electrophoresis, the gel was removed from the Electrophoresis cell and placed into a Gel frame to facilitate staining. The gel was placed into fixative solution (60 % ethanol v/v, 30 % distilled H_2O v/v, 10 % glacial acetic acid v/v) for 5 min. After fixing, the back of the gel was wiped to remove excess fixative, and the gel was dried in an incubator at 40°C until completely dry (~ 30 min). The dried gel was then processed in the following solutions in order: Lipoprotein working stain (Sudan Black B stain, 0.07 %) for 5 min; Destain solution I (45 % v/v ethanol, 55 % v/v

distilled H_2O) for 3 dips; Destain solution II (as Destain I) for 3 dips; Destain solution III (as Destain I) for 5 min. The gel was rinsed in distilled H_2O, and placed in an incubator until fully dried, as detailed above.

Results are expressed as relative electrophoretic mobility (REM), the migration of sample LDL relative to the mobility of the LDL band in normal serum. Normal control serum was electrophoresed in conjunction with sample LDL on each individual gel.

2.3.19 Immunohistochemistry

For the studies investigating the cellular localisation of TR (section 3.2.5 and 4.2.3), immunohistochemistry was employed. Briefly, HaCaT cells, human primary keratinocytes, EAhy926 cells, and HUVEC were grown to approximately 70 % confluence on 22 x 22 cm sterile glass coverslips in 6- well culture plates. Each respective culture medium was 'Se-deficient 'unless stated. The medium was aspirated from the cells, and each well washed twice with 4 ml PBS. The glass coverslips were then removed from the culture plates into a shallow dish of acetone for 2-3 min to fix the cells. After fixing, the glass coverslips were rinsed with fresh PBS, and placed back into the wells of the original culture plates which had been filled with absolute ethanol. The cells remained preserved in this state at 4°C until immunohistochemistry took place.

Immunohistochemistry for TR was carried out by Mr Craig Walker, Department of Dermatology, University of Edinburgh. Staining took place in a humidity chamber, with the coverslips placed 'cell side down' onto a microscope slide primed with 100 μl of the respective reagent.

The fixed coverslips were transferred into a coverslip staining rack and rinsed via immersion in two changes of Tris buffer containing 0.1 % BSA. The coverslips were placed in a humidity chamber at RT, where they were treated with 20 % normal swine serum (NSS) for 10 min. The NSS was then drained from the coverslips and replaced with rabbit anti-human TR antibody at a dilution of 1:200. Incubation with the TR antibody was for 16 hr at 4°C. Non-immune rabbit serum was used as a negative control in place of the primary antibody.

The coverslips were then washed twice for 5 min in Tris buffer. The secondary antibody used was biotinylated swine anti-rabbit immunoglobulins, diluted 1:400. Treatment with the secondary antibody was for 30 min at RT, after which the coverslips were washed twice for 5 min in Tris buffer. Avidin-Biotin Complex Alkaline Phosphatase was applied to the coverslips for 30 min, after which the coverslips were again washed twice for 5 min in Tris buffer. Visualisation was with freshly-prepared NBT/BCIP (Nitro-Blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate) at a 1:50 dilution (in 0.1 M Tris, pH 9.5; 50 mM $MgCl_2$; 0.1 M NaCl) for 1 hr at RT. After this time, the coverslips were washed thoroughly in tap H_2O and

mounted onto microscope slides using a small drop of Aquamount. The slides were examined under a Leitz Ortholux 2 microscope fitted with a Leica HBO 50W mercury vapour lamp using a Kodak Wratten No. 47B excitation filter and a Kodak Wratten No.12 barrier filter. The cells were photographed with a Leitz Vario Orthomat 2 camera using an Ektachrome 400 film.

2.3.20 Ultraviolet irradiation of cells *in vitro*

Prior to irradiation with UVB, HaCaT cells were grown to 70 % confluence in 24 well culture plates. Before irradiation the medium was removed from the wells into sterile containers to be replaced after irradiation. The cells in each well were then washed twice with 1 ml PBS, and covered with 0.5 ml PBS per well for the irradiation procedure. Cells were irradiated in their tissue culture plates without the lids on, with broadband UVB from a bank of two TL-20W/12 lamps (Philips, Croydon, UK) with an output range of 270-350 nm (peak at 308 nm). The irradiation dose was 80 mW/cm^2 at a vertical distance of 30 cm from the tubes (Kondo *et al.*, 1993). An IL-1400A radiometer with a SEL240/UVB 1/TD UVB detector (International Light Inc., Montreal, Canada) with a spectral sensitivity range of 280-320 nm was used to quantify the UVB dose in Joules per square metre (J/m^2) output that the cells received.

The output of the lamps was determined by Dr Neil Gibbs of the Photobiology Unit, Ninewells Hospital, Dundee, using a spectroradiometer (model 742, Optronic Laboratories) across the spectral range 250 – 400 nm. The tube target distance was 16 cm. The spectral output is shown in figure 2.04.

Cell viability following UVB irradiation was assessed either by trypan blue assay (section 2.3.14) or LDH assay (section 2.3.13).

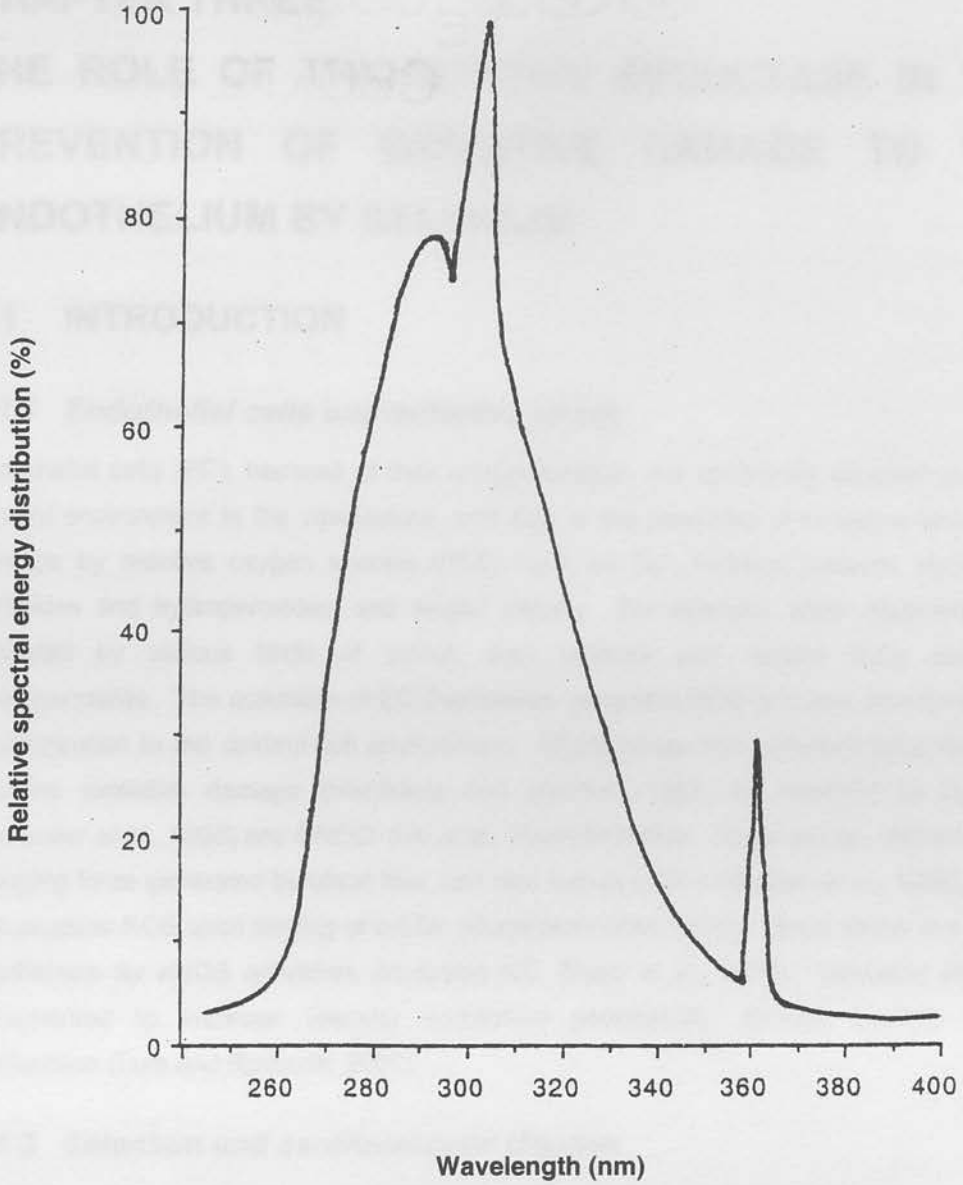


Figure 2.04 Emission spectrum for TL-20W/12 UVB lamps. The output of the lamps was determined using a spectroradiometer (model 742, Optronics Laboratories) across the spectral range 250 – 400 nm. The tube target distance was 16 cm.

CHAPTER THREE
THE ROLE OF THIOREDOXIN REDUCTASE IN THE PREVENTION OF OXIDATIVE DAMAGE TO THE ENDOTHELIUM BY SELENIUM

3.1 INTRODUCTION

3.1.1 Endothelial cells and oxidative stress

Endothelial cells (EC), because of their unique location, are continually exposed to a pro-oxidant environment in the vasculature, and thus to the possibility of oxidative stress and damage by reactive oxygen species (ROS) such as $O_2^{\bullet-}$, hydroxyl radicals, H_2O_2, lipid peroxides and hydroperoxides, and singlet oxygen. For example, when neutrophils are activated by various kinds of stimuli, they produce and release H_2O_2 and lipid hydroperoxides. The activation of EC themselves generates ROS and may therefore make a contribution to the oxidant-rich environment. Shear stress and turbulent blood flow can produce oxidative damage (Hishikawa and Lüscher, 1997) by induction of $O_2^{\bullet-}$ (De Keulenaer *et al.*, 1998) and $ONOO^-$ (Go *et al.*, 1999) formation. Shear stress, created by the dragging force generated by blood flow, can also induce LOX-1 (Murase *et al.*, 1998), which can produce ROS upon binding of oxLDL (Cominacini *et al.*, 2000). Shear stress is a critical mechanism for eNOS activation, producing $NO^{\bullet}$ (Patel *et al.*, 2000). Oxidative stress is documented to increase vascular endothelial permeability, thereby causing barrier dysfunction (Lum and Roebuck, 2001).

3.1.2 Selenium and cardiovascular disease

Oxidative damage to the endothelium is one of the principle mechanisms in the pathogenesis of atherosclerosis (Gimbrone, 1995; McGorisk and Treasure, 1996; Ross, 1993). Cytoprotection against oxidative damage is accomplished by an array of enzymatic and non-enzymatic antioxidant systems. In the endothelial cell these systems consist principally of antioxidant vitamins, superoxide dismutase (SOD), catalase and selenoproteins such as the glutathione peroxidases (GPX) and possibly thioredoxin reductase (TR).

Low plasma Se levels in humans have been associated with an increased risk of cardiovascular disease, including coronary atherosclerosis (Kok *et al.*, 1991; Nève, 1996; Salonen *et al.*, 1982; Suadicani *et al.*, 1992). Se deficiency can result in an accumulation of fatty acid peroxides in the heart and lead to the formation of substances that enhance

formation of clots (Masukawa *et al.*, 1983). Cells cultured under Se-deficient conditions accumulate LOOHs more rapidly under conditions of oxidative stress than control cells, and die more rapidly (Geiger *et al.*, 1993; Lin *et al.*, 1992). Se-deficiency increases the susceptibility of BAEC to cytotoxic damage by *tert*-butylhydroperoxide- (t-BuOOH) (Hara *et al.*, 2001).

Resistance to oxidative damage is conferred on EC *in vitro* that receive Se supplementation (Ochi *et al.*, 1992; Thomas *et al.*, 1993). Ochi *et al.* demonstrated a dramatic decrease in the toxicity of the hydroperoxide (15*S*)-hydroperoxy-(5*Z*), (8*Z*), 11(*Z*), 13(*E*)-eicosatetraenoic acid ((15*S*)-HPETE) (which occurs *in vivo* by oxygenation of arachidonic acid by arachidonic acid 15-lipoxygenase) (Björnstedt *et al.*, 1995) using sodium selenite and ebselen (a synthetic glutathione peroxidase mimic) pre-incubations (Ochi *et al.*, 1992). These findings, and subsequent investigations manipulating the glutathione redox cycle, suggest that (15S)-HPETE-induced cytotoxicity could be attributed to decreased GPX activity.

In agreement with Ochi *et al.*, Thomas *et al.* proposed that an observed decrease in both t-BuOOH and photogenerated oxidized low density lipoprotein (oxLDL)-mediated cytotoxicity to Se-supplemented BAEC resulted from an increased expression of both cytoplasmic glutathione peroxidase (cyGPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX) (Thomas *et al.*, 1993).

3.1.3 Selenoenzymes and the cardiovascular system

The involvement of other selenoenzymes such as TR has yet to be fully investigated in such a protective role, but human EC express very high concentrations of TR. The above-mentioned studies used bovine EC; there have been some studies of cytotoxicity and protection in human EC (Cho *et al.*, 1999; Harlan *et al.*, 1984; Varani and Dame, 1995), but none using Se. Bovine cells may be a poor model for human EC, however, since TR may not be as important for antioxidant protection as it is in human EC.

Differences in the expression of intracellular selenoproteins occur between EC isolated from different vascular beds, and clear differences exist in selenoprotein expression in EC of different species. It is possible, therefore, that the antioxidant systems of EC may vary between cells isolated from different vascular beds and different species. Extracellular SOD in the aortic wall is known to vary widely between species (Strålin *et al.*, 1995). BAEC contain significantly higher activity of SOD and catalase than rabbit aortic EC (Steinbrecher, 1988).

The GPXs are considered to exert powerful antioxidant function in the cell cytoplasm (Brigelius-Flohé, 1999), and the expression of the GPXs can be increased in situations of oxidative stress (de Haan *et al.*, 1998; Mitchell *et al.*, 1996). cyGPX catalyses the reduction of a variety of hydroperoxides including hydrogen peroxide, cumene hydroperoxide, t-BuOOH, and fatty acid hydroperoxides (Flohé, 1989; Rotruck *et al.*, 1973). In contrast, PHGPX has been shown to catalyse the reduction of fatty acid hydroperoxides and cholesterol hydroperoxides (Ursini *et al.*, 1985). In its role of reducing hydroperoxo-groups in complex lipids, PHGPX is synergistically supported by tocopherols, which reduce lipid peroxy radicals to lipid hydroperoxides. The latter, if not reduced by PHGPX, would re-initiate lipid peroxidation. A tumour cell line that over-expresses PHGPX has been found to be more resistant to cell death induced by photochemically-generated cholesterol hydroperoxides than wild-type or null-transfected cells (Hurst *et al.*, 2001).

The effect of Se supplementation on the heart is primarily reflected in an increase of PHGPX activity, and not of cyGPX (Jotti *et al.*, 1994). This finding supports the concept that the physiological response to the cytotoxic action of ROS generated during redox-cycling of quinone-containing compounds such as doxorubicin (adriamycin) is directed towards maintenance of cell membrane integrity and prevention of lipid peroxidation.

cyGPX knockout mice have been developed as a model to study the role of cyGPX in normal physiology and in the pathogenesis of a number of disease states. Using this model, mice deficient in cyGPX develop normally and show no increased sensitivity to pulmonary hyperoxia (Ho *et al.*, 1997). The lack of any overt phenotype in these knockout mice is unsurprising considering the clinical phenotype associated with patients having deficiencies in GSH regeneration. Such patients are normal so long as they are unchallenged with hydroperoxides (Beutler, 1983).

However, the contribution of cyGPX as an antioxidant defence mechanism against the pathogenic effect of ROS in other conditions has not been ruled out. For example, oxidative stress induced by 30 mg/kg of paraquat, which is approximately LD_{20} for mice, killed 100 % of cyGPX knock-out mice, whereas control mice showed no signs of toxicity at this dose (de Haan *et al.*, 1998). In the same study neurons from cyGPX knock-out mice were more sensitive to oxidative stress induced by H_2O_2.

Despite ongoing attempts to produce a PHGPX knockout mouse, a successful model has not yet been obtained. Such a model would be useful to provide further information on the role of PHGPX.

TR is a dominantly expressed selenoprotein in both HUVEC and HCAEC. TR acts as an antioxidant either directly or through the action of thioredoxin, and can reduce and detoxify lipid hydroperoxides, H_2O_2 and organic hydroperoxides, and peroxynitrite directly using NADPH as a cofactor (Arteel *et al.*, 1999; Björnstedt *et al.*, 1995; May *et al.*, 2002). In addition, TR is able to regenerate bioactivity in proteins inactivated by oxidative stress (Spector *et al.*, 1988) (Ejima *et al.*, 1999), can regenerate ascorbic acid from dehyroascorbate (May *et al.*, 1997), and can induce manganese superoxide dismutase (Das *et al.*, 1997). TR is reported to have a superior capacity for detoxifying H_2O_2 and lipid peroxides over that of the GPXs (Björnstedt *et al.*, 1995). This, together with the high expression of TR in human EC, suggests that TR may be more important than the GPXs at protecting cells from oxidative damage. TR may also contribute to the protective effects of Se against oxidative damage to the endothelium since the TR/Trx system can maintain NOS in a reduced configuration potentially overcoming the hypothesised oxidative deactivation of NOS (Patel *et al.*, 1996).

The upregulation of selenoprotein expression in cultured cells through Se-supplementation is widely reported (Buckman *et al.*, 1993; Dreher *et al.*, 1998; Fujiwara *et al.*, 1999; Ricetti *et al.*, 1994; Stewart *et al.*, 1999; Takahashi *et al.*, 1986; Thomas *et al.*, 1993; Yarimizu *et al.*, 2000). It is therefore possible that decreased expression and activity of these selenoproteins in Se-deficiency in man may increase the susceptibility of the endothelium to oxidative damage.

Labelling with [^{75}Se]-selenite provides a reliable, precise and sensitive method to assess selenoprotein expression in tissues and cells. The [^{75}Se] is specifically directed to and incorporated into selenocysteine residues via a UGA codon. However, since equilibration of exogenous [^{75}Se]-selenite with the endogenous pool of Se and selenoproteins can take within excess of 27 hr (Beech *et al.*, 1994), cells are usually labelled for between 32 and 48 hr to ensure that a steady state of labelling has been achieved.

3.1.4 Oxidised LDL and the endothelium

OxLDL is an important mediator of oxidative damage to the endothelium *in vivo* (Nielson, 1999; Steinberg, 1991; Witzum and Steinberg, 1991; Witzum and Steinberg, 2001), and oxidative modification of LDL cholesterol plays a central role in atherogenesis (Berliner *et al.*, 1995; Diaz *et al.*, 1997; Steinberg, 1999; Steinberg *et al.*, 1989). OxLDL activates PKC in HCAEC *in vitro* (Li *et al.*, 1998); activation of PKC, by the phorbol ester PMA, in HUVEC decreases TR expression as assessed by [^{75}Se]-labelling of cells and Western blot analysis (Anema *et al.*, 1999). The activation of PKC through oxLDL has been implicated in vascular disease (Ohgushi *et al.*, 1993). Since TR may provide essential antioxidant defence to the

EC, down-regulation of TR by this mechanism may diminish the antioxidant capacity of the EC, leaving the cell vulnerable to oxidative damage and to development of atheroma. α-Tocopherol provides resistance to PKC stimulation by oxLDL and PMA in cultured human aortic EC (Keaney *et al.*, 1996). However treatment of HUVEC with the phorbol ester phorbol 12, 13-dibutyrate induced a 2-fold increase in cyGPX mRNA levels (Jornot and Junod, 1997), and treatment with PMA increases cyGPX expression (Miller, 2000).

Upon oxidation by metal ions or by cells *in vitro*, a variety of oxidation products are formed from the peroxidation of the lipid constituents of LDL, and the oxidative deterioration of apoprotein B (Rosenfeld, 1991). Each of the lipid classes can be oxidised, including sterols, fatty acids in phospholipids, cholesterol esters, and triglycerides. During oxidation of LDL, the various lipid classes undergo modification. This includes the oxidation of cholesterol to oxysterols, oxidation of PUFA to form various aldehydes, including 4-hydroxynonenal, and the conversion of phospholipids to lysophospholipids by a phospholipase A_2-like activity (Colles *et al.*, 1996). The cytotoxicity of oxLDL to EC has been demonstrated in numerous studies (Claise *et al.*, 1997; Thérond *et al.*, 2000; Thomas *et al.*, 1993). OxLDL shows cytotoxic effects on EC affecting several functions (Coffey *et al.*, 1995; Escargueil-Blanc *et al.*, 1997; Thomas *et al.*, 1993). However previous studies in human EC have not explored the ability of Se to prevent oxLDL cytotoxicity.

Endothelial cells *in vitro* are documented to oxidise LDL (Dugas *et al.*, 1998; Fernando *et al.*, 1993; Henriksen *et al.*, 1981; Nagelkerke *et al.*, 1984a; Nagelkerke *et al.*, 1984b; Parthasarathy *et al.*, 1989; Steinbrecher, 1988; Steinbrecher *et al.*, 1984; van Hinsberg *et al.*, 1986; Wilkins and Leake, 1994). Few studies have investigated the role of cell-associated antioxidants in LDL modification, as opposed to LDL-associated or extracellular antioxidants. These studies have shown that cultured vascular cells supplemented with ascorbate, α-tocopherol, β-carotene, or probucol exhibit a lowered capacity to modify LDL (Martin and Frei, 1997; Navab *et al.*, 1991; Parthasarathy, 1992; Reaven *et al.*, 1994; Steinbrecher *et al.*, 1984).

Assessment of the effect of Se supplementation of EC on their ability to promote LDL oxidation has not been studied to date. Ebselen has been shown to prevent LOOH formation during oxidation of LDL by Cu^{2+}, and also inhibited formation of LOOH and spared α-tocopherol during oxidation of LDL mediated by peroxyl radicals (Lass *et al.*, 1996).

Studies using oxLDL are complicated by the considerable variation in the products formed between different preparations. The products formed, and the extent of the changes in the LDL, are dependent on several factors including; the cell type used to initiate oxidation, the

metal ion concentration in the medium as well as the composition of the medium, the incubation conditions (e.g. length of time of exposure to pro-oxidant conditions) and the inherent susceptibility of the native LDL to be oxidised in the peroxidation conditions used for different studies (Esterbauer and Jürgens, 1993; Kuzuya *et al.*, 1991; Witzum and Steinberg, 1991) as well as isolation procedures (Parthasarathy *et al.*, 1999). In addition, marked variations in the degree to which LDL preparations can be modified have been observed with LDL from various donor individuals (van Hinsberg *et al.*, 1986) possibly due to differences in levels of PUFA, ubiquinol, and free cholesterol (Kontush *et al.*, 1996).

Therefore, there is no unique LDL particle corresponding to 'oxidised LDL', but rather there is a broad spectrum of 'oxidised LDLs'. Thus, the toxic composition of any one batch of oxLDL made using LDL isolated from different sources, even when using constant conditions is known to vary, which can make interpretation of results difficult.

t-BuOOH is a widely-used model toxicant (Elliot *et al.*, 1995; Hara *et al.*, 2001; Nardini *et al.*, 1998; Schuppe *et al.*, 1992; Thomas *et al.*, 1993), which is a redox-cycling agent (Comporti, 1989). The compound causes cytotoxicity, following reduction itself by intracellular reductases, via oxygen reduction (predominantly one-electron reduction) and consequent formation of ROS (Comporti, 1989). t-BuOOH has the advantage of not being subject to the inherent problem of between-batch variation which exists when oxLDL is used as an oxidative stress-inducing agent. However, it could be argued that t-BuOOH is not an agent that occurs naturally, unlike oxLDL. In addition, t-BuOOH is a substrate for cyGPX and PHGPX, but cyGPX is at least 10-fold more active than PHGPX on t-BuOOH (Geiger *et al.*, 1993), and thus may bias the mode of detoxification towards the GPX enzymes. This study encompassed experiments using both oxLDL and t-BuOOH. Cytoprotection studies using sodium selenite were performed using both oxLDL and t-BuOOH, whilst investigation of the individual selenoproteins responsible for the protection utilised t-BuOOH for all studies except one due to an unlimited supply of t-BuOOH.

Conceptualized forms of oxLDL

LDL at varying stages of oxidation is used in studies of cytotoxicity and protection, and LDL receptor studies. Although various groups have designated the LDL used as either 'minimally modified LDL' (mmLDL) or 'oxidised LDL' (oxLDL), there are a variety of oxidation processes used. An extensive variety of normal and pathological conditions may occur to oxidise intrinsic LDL *de novo*. When such oxidation is conducted to a minimal degree, the resultant particle may characterise mmLDL. This LDL form may be undistinguishable (physically) from the native LDL, apart from the loss of antioxidants and PUFA. The apoB-100 is intact, and minor protein damage or modification has occurred. In contrast, the lipids

are affected to a large degree. mmLDL has low levels of TBARS and is taken up by specific LDL receptors.

When LDL is oxidised, by copper for example, it undergoes oxidation after an initiation, propagation and termination sequence (Esterbauer *et al.*, 1992). At the point when oxidation reaches a plateau, all the oxidisable fatty acids are consumed, and the particle is abundant in oxidised fatty acids. A fully oxidised LDL particle will have extensive proteolysis, oxidation of amino acids, cross-linking, and modification of the amino acid groups of the apoB. Such a particle is unlikely to occur *in vivo*, and would probably be cleared from plasma by the liver (Parthasarathy *et al.*, 1999).

The most common method for the initiation of oxidation of LDL *in vitro* is incubation in the presence of Cu (II) ions. Lipid peroxidation in LDL can be initiated by adding Cu (II) ions, which are suggested to participate in redox cycling reactions with endogenous or contaminating lipid hydroperoxides (Burkitt, 2001).

3.1.5 Homocysteine and endothelial cells

Hyperhomocysteinemia has been described as an independent risk factor for endothelial dysfunction and the subsequent development of atherosclerosis (Outinen and Austin, 2000; Outinen *et al.*, 1999; Outinen *et al.*, 1998; Upchurch *et al.*, 1997). Hyperhomocysteinemia is believed to injure EC *in vivo* through a number of mechanisms, including generation of H_2O_2 and $O_2^{\bullet-}$ via auto-oxidation of homocysteine (Hcy) (Weiss *et al.*, 2001). The increased H_2O_2 accumulation may result from the ability of Hcy to down-regulate the expression of cyGPX (Upchurch *et al.*, 1997). Hcy also decreases the expression of SOD as well as cyGPX in HUVEC (Outinen *et al.*, 1999). Homocysteinemia may also affect endothelial dysfunction by decreasing the production and/or bioavailability of $NO^{\bullet}$ (Upchurch *et al.*, 1997; Weiss *et al.*, 2001). The $O_2^{\bullet-}$ formed via autooxidation of Hcy may react with $NO^{\bullet}$ to produce $ONOO^-$, leading to oxidative inactivation of $NO^{\bullet}$ with the resultant endothelial dysfunction. Over-expression of cyGPX in mice protects against Hcy-induced endothelial dysfunction (Weiss *et al.*, 2001). Other studies have linked decreased GPX levels with an increased incidence of coronary artery disease. For example, platelet GPX activity is impaired in patients with coronary artery disease (Guidi *et al.*, 1986). However, a study in 10 patients with inherited defects of Hcy metabolism showed that those individuals with elevated plasma Hcy (> 20 μM) had increased SOD and cyGPX activities (Moat *et al.*, 2000).

3.1.6 Endothelial cell models

The use of cell culture has allowed the study of homogenous cell populations uncomplicated by the confounding factors of other tissues. Large vessel endothelial cell culture is a well-

established model for the study of the endothelium. The human umbilical vein is often the chosen vessel for the study of human endothelial function as it has several advantages as a source of EC. It is a non-branching vessel with a large intimal surface area making technical isolation of cells easy. However, many variables can affect the viability of isolated cells, including foetal stress, maternal anaesthesia, smoking and other toxins (Gimbrone *et al.*, 1974; Tu *et al.*, 1994). The use of HUVEC is also complicated by the genetic variability between preparations, limited population doublings, difficulty in culture and the requirement for specialised growth factors. In addition, a varied susceptibility to toxic agents between different isolates of HUVEC, and different passage number within the same isolate, has been reported (Harlan *et al.*, 1984).

Human coronary arterial EC (HCAEC) may be a good model for human EC as the coronary artery is one of the principal vascular sites affected by the formation of atheroma. In addition, HCAEC have a similar selenoenzyme profile of expression to HUVEC. However, HCAEC are not as easy to culture as HUVEC, are technically more difficult to isolate due to their anatomical source, and obtaining donor material for preparations is problematic.

An alternative to the use of HUVEC or HCAEC is to use human transformed cell lines such as EAhy926. EAhy926 is an endothelial cell line established by hybridising primary HUVEC with A549 human lung tumour cells (Edgell *et al.*, 1983). Cells in primary culture have a limited replication potential and show a tendency to senesce in culture (Schwartz, 1978), whilst EAhy926 cells retain many of the differentiated functions common to primary EC beyond 100 passages. These functions include the expression of von Willebrand Factor (Edgell *et al.*, 1983), prostacyclin formation (Suggs *et al.*, 1986) and expression of endothelin-1(Saijonmaa *et al.*, 1991). The selenoprotein profile of EAhy926 cells has not been previously determined, which is essential in order to establish whether this more convenient cell line would provide a suitable model for future studies of selenoprotein expression in EC.

Endothelial cells isolated from a number of different species have been used as model systems to investigate human pathologies. For example bovine aortic EC (BAEC) were used in the earliest studies due to their ease of isolation and subcultivation. When initially isolated and characterised, porcine aortic EC (PAEC) (Slater and Sloan, 1975) were thought to provide a suitable alternative to HUVEC based on similarities of the porcine cardiovascular system with that in man. In addition porcine aorta is subject to atheroma formation and should provide a useful model for the study of atherosclerosis (Rosenthal and Gotleib, 1990). However, the source of EC used for culture is extremely important because responses and properties of cells of different species vary considerably. For example PAEC,

unlike HUVEC and BAEC, do not have Factor VIII-related antigen immunoreactivity (Rosenthal and Gotleib, 1990).

As well as species differences in the properties of EC it has also been shown that EC isolated from different sites in the vasculature exhibit different properties. For example, EC derived from human dermal microvasculature (HMVEC) form capillary-like tubes at a faster rate than HUVEC, and fibroblast growth factor stimulates increased production of tissue plasminogen activator (t-PA) in aortic EC but inhibits t-PA production in HUVEC (Halliday *et al.*, 1998). HMVEC do not show an age-dependent decrease in sensitivity to killing by activated neutrophils that is a characteristic function of HUVEC (Varani and Dame, 1995; Varani *et al.*, 1992). Arterial and venous EC show differences in the production of angiotensin-converting enzyme (Johnson, 1980) and their response to cytokine stimulation (Hauser *et al.*, 1993). These observations have led to the suggestion that HUVEC, despite being used by a number of researchers in the field of vascular disease (Jornot and Junod, 1997; Kvietys and Granger, 1997; Milner *et al.*, 1990; Zhao *et al.*, 1997), may not be the most suitable model in the study of human cardiovascular disease. Indeed a more suitable model to study vascular disease may be EC isolated from arterial vascular beds as opposed to venous; thus HCAEC may provide a good model as the coronary artery in particular is one of the main vascular beds affected by the formation of atherosclerotic lesions.

If the efficacious effect of Se on EC function is to be studied, it is essential that a model system be chosen which reflects the selenoprotein expression and function of EC which line vessels prone to developing vascular disease. In addition, although previous work on Se and BAEC is of importance, it would be valuable to determine if Se can exert a similar protective response in human EC.

Unstimulated EC in culture can generate superoxide (Rosen and Freeman, 1984) and H_2O_2 (Sundqvist, 1991). This suggests that cultured EC may be under oxidative stress. This may be an important point to consider for cytoprotection studies since conventional culture media have been reported to be deficient in antioxidants (Baker *et al.*, 1998; Brigelius-Flohé *et al.*, 1995; Leist *et al.*, 1996) (see section below on culture media). EC *in vivo* are exposed to a maximum PO_2 of 80-90 mm Hg (10.65 – 11.98 KPascal) (arterial), and a minimum PO_2 of 40 mm Hg (5.33 KPascal) (venous). In comparison, EC in culture are typically maintained at an ambient PO_2 of 140 mm Hg (18.64 KPascal) (Kvietys and Granger, 1997). The magnitude of this oxidative stress may alter with varying culture conditions. For example, the EC content of antioxidant enzymes (catalase, SOD, and GPX) varies as a function of passage number (Bishop *et al.*, 1985; Hart *et al.*, 1985; Varani *et al.*, 1992). The cyGPX activity and catalase activity significantly decrease after the first subculture of bovine pulmonary artery EC (Hart *et*

al., 1985). Glutathione levels decrease in HUVEC with passage number (Tu *et al.*, 1994). This variation in antioxidant status with EC age in culture is, however, unpredictable, i.e. some enzymes increase with age while other enzymes decrease. As the age of HUVEC in culture increases, the intracellular iron, which facilitates production of ROS, decreases (Varani *et al.*, 1992), thereby rendering the cells more resistant to oxidative damage. Thus, in any EC model system in which oxidative stress is being studied, it is important to consider the antioxidant status of the cells (in their 'natural' state).

3.1.7 Culture media and selenium

To study the ability of Se to protect EC against oxidative damage from t-BuOOH, EAhy926 cells were cultured and maintained as described in section 2.3.2. Prior to each experiment, the cells were sub-cultured and seeded into 24-well culture plates in high glucose (4.5 g/L) Dulbecco's Modified Eagles medium (DMEM) containing 10 % FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, and 0.8 mM thymidine, in a humidified atmosphere of 5 % CO_2, 95 % air at 37°C. The Se content of the DMEM basal medium and the FBS used for supplementation was determined by acid digestion followed by fluorimetric analysis (section 2.3.16). DMEM basal medium had a Se concentration of 0.351 nM, and was therefore classified as Se-deficient medium. FBS (undiluted) had a Se concentration of 13.78 nM, although this form of Se is unlikely to be bioavailable to the cells (see below). The Se content of basal culture media varies between types of media; for example, basal DME medium for mouse hybridomas contains 1 nM (0.001 μM) Se, but RPMI 1640 medium for human hepatoma cells contains 0.03 μM Se (Freshney, 1992). The Se content of FBS can also vary greatly, with values ranging from zero to 0.2 μM (Leist *et al.*, 1996), 0.48 μM (Fujiwara *et al.*, 1999), and between 0.16 and 0.18 μM (Geiger *et al.*, 1993). Skin cells in culture with media of differing basal Se levels supplemented with sodium selenite or selenomethionine are afforded similar levels of protection against UVB-mediated oxidative stress with the same selenite and selenomethionine concentrations despite the differing Se content of the basal media (Rafferty, 2000); this may indicate that the Se in the basal medium is not bioavailable for use by the cells. Se contained in conventional culture media is predominantly incorporated into the proteins of serum supplements, and as such is not easily utilised by cells in culture (Brigelius-Flohé *et al.*, 1995; Marcocci *et al.*, 1997). Conventional culture media require Se supplementation in order to guarantee selenoprotein synthesis in cultured cell lines (Baker *et al.*, 1998; Brigelius-Flohé *et al.*, 1995).

HAEC in culture under standard conditions are deficient in vitamin C, and as a result may be under increased oxidative stress (Smith *et al.*, 2002). Deficiency of vitamin C in HAEC lead to a compromised intracellular thiol redox status (GSH/GSSG ratio), and a higher rate of production of ROS, both of which were attenuated by repletion with vitamin C. Standard

culture media for HAEC do not routinely contain vitamin C since it is unstable in aqueous solution.

The studies reported in this chapter aimed to:

- determine the suitability of the cell line EAhy926 as an appropriate model in which to investigate the role of Se and selenoproteins in human endothelial cells
- examine the ability of sodium selenite supplementation to protect EAhy926 cells from cytotoxicity resulting from exposure to oxidised lipids, and associate any observed protection with changes in the expression and activity of TR and the activity of cyGPX and PHGPX
- investigate the importance of TR, cyGPX and PHGPX in the protection of EAhy926 cells from toxicity of oxidised lipids by use of gold thioglucose-mediated inhibition of selenoenzyme activity

3.2 MATERIALS AND METHODS

3.2.1 General methods for cytotoxicity and selenoenzyme expression studies

In all cytotoxicity studies using endothelial cells, the cells were passaged into 24-well culture plates using Se-deficient medium, with all test conditions in triplicate wells of confluent cells, unless otherwise stated. After an incubation in the presence of t-BuOOH or oxLDL, both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.13. Details on the Se content of cell culture media are described in section 3.1.7.

For all studies of selenoprotein expression, endothelial cells were passaged into 75 cm^2 flasks, with all test conditions in triplicate flasks, except control cells which were grown in quadruplicate. Following the incubation, the medium was sampled for LDH assay to check for cytotoxicity, and the cells were washed twice with 10 ml EBSS, and harvested via scraping into 20 ml EBSS. Efficiency of harvesting was determined by light microscopy. The cells were then pelleted by centrifugation at 500 *x g* for 10 min. The EBSS was aspirated, and the pellets frozen at - 80°C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed and lysed by sonication (three pulses of 10 sec using a Soniprep 150 Sonicator) on ice in 0.125M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1 % Triton X-100 (peroxide- and carbonyl-free). The levels of the selenoenzymes were then determined as detailed in sections 2.3.5.1, 2.3.6, 2.3.7 and 2.3.8.

3.2.2 Intracellular [^{75}Se]-selenoprotein expression profile in EAhy926 cells and HUVEC

The intracellular selenoprotein profile of the human endothelial cell line EAhy926 was compared to that of HUVEC. 75 cm^2 flasks of both HUVEC and EAhy926 cells were seeded and maintained in EGM-2 and DMEM containing 10 % FBS and 1 % HAT respectively as described in sections 2.3.2 and 2.3.3. At confluence the cells were labelled with [^{75}Se]-selenite (0.02 MBq/ml). After 48 hr incubation, the cells were harvested into 20 ml EBSS by scraping, and centrifuged at 2000 *x g* for 10 min at 4°C. The resulting cell pellet was re-suspended in 200 μl 60 mM Tris buffer, pH 7.4 (4°C), containing 1 mM EDTA and 1 mM dithiothreitol (Tris buffer). The cells were lysed by sonication on ice for 30 sec (three ten second pulses) using a Soniprep 150 Sonicator.

Protein concentrations were measured using the Bradford assay (section 2.3.9) and the samples were diluted to a common protein concentration with Tris buffer. The cell lysates were prepared for separation by SDS-PAGE (section 2.3.10). The [^{75}Se]-labelled proteins

present in 25 μg of protein from each of the cell types were separated on a single SDS-PAGE gel (section 2.3.10) to allow a direct comparison between the two cell types. The resulting gel was dried and the [^{75}Se]-labelled selenoproteins visualised by autoradiography (section 2.3.11).

The SDS-gels were scanned using an EpsonGT-9500 to create a digitalized image. The radioactivity in each band was quantified using the Phoretix software. SDS-PAGE for this experiment was carried out by Mrs S Miller of the Department of Clinical Biochemistry, The University of Edinburgh.

3.2.3 Intracellular selenoprotein expression and activity of different vascular endothelial cells

HUVEC, EAhy926 cells, HCAEC and BAEC were isolated and/or maintained as previously described (see sections 2.3.2 and 2.3.3).

For measurement of TR concentration and activity, the cyGPX activity and the PHGPX activity (sections 2.3.6, 2.3.5.1, 2.3.7 and 2.3.8), triplicate 75 cm^2 flasks of each cell type was grown and maintained as previously described (2.3.2 and 2.3.3). At confluence the cells were harvested and lysed as detailed in section 3.2.1. The cell lysates were subsequently frozen at - 80°C until assay. All the samples for each measurement were analysed in the same assay to avoid any between-assay variation.

3.2.4 The effect of sodium selenite supplementation on intracellular selenoprotein expression and activity in HUVEC, HCAEC, BAEC and EAhy926 cells

EAhy926 cells were passaged into 75 cm^2 flasks and grown to 70 % confluence. The cells then received medium containing 0, 1, 10, 40, 50, 100, 200 or 1000 nM sodium selenite for an incubation of 48 hr. Harvesting was carried out as described in section 3.2.1. TR activity, cyGPX and PHGPX activity were determined as described in sections 2.3.5.1, 2.3.7 and 2.3.8, respectively.

For measurement of TR concentration and activity, cyGPX and PHGPX activity, HUVEC, HCAEC and BAEC were passaged and seeded at a density of approximately 3000 cells/cm^2 into 75 cm^2 flasks for selenoprotein measurement. The cells were cultured and maintained in Se-deficient medium to which selenite had been added at concentrations ranging from 1 to 1000 nM for HUVEC, and from 1 to 160 nM for HCAEC and BAEC. The culture medium was removed and replaced with fresh medium containing sodium selenite on every other day. Upon reaching full confluence (approximately 9 to 12 days) the cells were harvested as described in section 3.2.1. TR concentration and activity, cyGPX and PHGPX activity were determined as described in sections 2.3.6, 2.3.5.1, 2.3.7 and 2.3.8, respectively. It was not

possible to determine TR expression in BAEC due to the lack of cross-reactivity of the antibody to human TR with the bovine protein. The cyGPX and PHGPX activity were not measured in all experiments due to a shortage of cells.

3.2.5 The cellular localisation of TR in EAhy926 cells and HUVEC

EAhy926 cells and HUVEC were grown to approximately 70 % confluence on 22 x 22 cm sterile glass coverslips in six well culture plates. Each respective culture medium was 'Se-deficient' unless stated otherwise. The medium was aspirated from the cells, and each well washed twice with 4 ml PBS. The glass coverslips were then removed from the culture plates and fixed in acetone. After fixing, the glass coverslips were rinsed with fresh PBS, and placed back into the wells of the original culture plates which had been filled with absolute ethanol. The cells remained preserved in this state at 4°C until immunohistochemistry took place, as described in section 2.3.19.

3.2.6 t-BuOOH cytotoxicity in EAhy926 cells cultured in selenium-deficient medium assessed by % LDH activity retention

The effect of a range of concentrations of t-BuOOH (0 – 325 μM) on % LDH activity retention by EAhy926 cells was determined. After a 20 hr incubation in the presence of t-BuOOH, both the medium and cells were harvested and analysed for % LDH activity retention (section 2.3.13).

3.2.7 The effect of cellular confluence level on susceptibility of EAhy926 cells to oxidative damage by t-BuOOH

Preliminary experiments to investigate the effect of different concentrations of t-BuOOH on LDH activity in EAhy926 cells cultured in Se-deficient medium showed great variability in the t-BuOOH concentrations seen to produce cell damage. One of the factors proposed to account for this observed variability was the degree of confluence of the cell monolayer. To investigate this, EAhy926 cells were passaged into 24 well plates and left to grow in Se-deficient medium until the required level of confluence was reached. The dose-response curve of t-BuOOH (0 – 125 μM and 0 – 400 μM respectively for the two experiments) on EAhy926 cells at differing confluence levels was determined. After a 20 hr incubation in the presence of t-BuOOH, both the medium and cells were harvested and analysed for % LDH retention (section 2.3.13). All plates of cells at differing confluence level received the same t-BuOOH solutions which had been prepared and stored at 4°C until further use.

To investigate the effect of the length of time between passage and treatment with t-BuOOH on the susceptibility of cells to cytotoxic damage, EAhy926 cells were passaged into 24-well plates, with one plate at double the seeding density of the other, such that both plates would

be of a similar confluence level when exposed to t-BuOOH (~100 % confluence). The cells were left to grow for either 2 days or 4 days after passage before exposure to t-BuOOH at a range of concentrations (0, 25, 50, 75, 100, 150, 200, 250 μM). Identical t-BuOOH solutions were used for both lots of cells, prepared on the day of first use, and stored at 4°C until required again. After a 20 hr incubation in the presence of t-BuOOH, both the medium and cells were harvested and analysed for LDH activity (section 2.3.13).

3.2.8 The ability of sodium selenite to protect against oxidative damage resulting from t-BuOOH exposure in EAhy926 cells

To investigate the possible protective effect of sodium selenite against oxidative damage mediated by t-BuOOH, EAhy926 cells were sub-cultured into 24 well plates using Se-deficient medium. The cells were then grown for 48 hr. Se-deficient medium to which a range of sodium selenite concentrations (0, 1, 10, 40, 50, 100, 200, 1000 nM) had been added was then placed on the cells. After an incubation period of 48hr, the cells were washed twice with 1 ml EBSS. Concentrations of 0 μM or 300 μM t-BuOOH were then added, prepared in Se-deficient medium, and left to incubate with the cells for 20 hr. After 20 hr both the medium and cells were harvested and analysed for % LDH retention as described in section 2.3.13.

3.2.9 Assessment of the direct effect of sodium selenite in the protection of EAhy926 cells against oxidative damage resulting from t-BuOOH exposure

To determine whether sodium selenite can exert a direct antioxidant effect against t-BuOOH-mediated cytotoxicity in EAhy926 cells, rather than through modification of selenoprotein expression, the following approach was utilised. EAhy926 cells were sub-cultured into 24 well plates using Se-deficient medium. After 48 hr, some cells received Se-deficient medium supplemented with 40 nM sodium selenite, whilst other cells continued to be maintained in Se-deficient medium. After an incubation period of 48 hr, all cells were washed twice with 1 ml EBSS. The cells then received Se-deficient medium supplemented with 40 nM sodium selenite simultaneously with the addition of a range of t-BuOOH concentrations (0 – 250 μM), or the same range of concentrations of t-BuOOH made up in Se-deficient, unsupplemented medium.

Control cells received no t-BuOOH or sodium selenite supplementation. After 20 hr both the medium and cells were harvested from all the culture plates and analysed for LDH activity as described in section 2.3.13.

3.2.10 Assessment of the effect of gold thioglucose on cyGPX activity, PHGPX activity and TR activity of EAhy926 cells

An experiment to investigate the timecourse of inhibition of TR activity by 10 μM gold thioglucose (GTG) was constructed to assess the optimal time for pre-incubation with GTG. EAhy926 cells were passaged into 75 cm^2 flasks and grown to 70 % confluence. The cells then received medium containing 10 μM GTG for an incubation of 24, 48 or 72 hr (triplicate flasks for each time point). Control cells received medium unsupplemented by GTG. The cells that received an incubation of 72 hr received fresh medium supplemented with 10 μM GTG after 48 hr. Following the incubation, the cells were washed twice with 10 ml EBSS, and harvested as detailed in section 3.2.1.

Once the time for pre-incubation had been optimised, the aim of these studies was to determine a concentration of GTG that would selectively inhibit only TR activity, without inhibiting the GPXs. Briefly, EAhy926 cells were passaged into 75 cm^2 flasks and grown to 70 % confluence. The cells then received medium containing 0, 1, 10, or 100 μM GTG for an incubation of 48 hr. Following the incubation, the cells were washed twice with 10 ml EBSS, and harvested as detailed in section 3.2.1. This experiment was performed twice. A further experiment using 0, 1.75, 2.5 μM GTG was performed in order to assess whether any of these concentrations of GTG was more selective in its inhibition of TR over that of cyGPX and PHGPX.

3.2.11 Assessment of the effect of gold thioglucose on the susceptibility of EAhy926 cells to oxidative damage resulting from t-BuOOH exposure

EAhy926 cells were passaged into 24 well plates using Se-deficient medium, and left to grow for 48 hr. After this time, the cells received normal unsupplemented medium, or the same medium containing 10 μM GTG for 48 hr. When the incubation was finished, the cells were washed twice with 1 ml EBSS, and fresh medium containing t-BuOOH (0, 75, 100 μM) was added for a 20 hr incubation. Both the medium and cells were harvested and analysed for % LDH retention as described in section 2.3.13. This experiment was then repeated using 1 μM or 10 μM GTG pre-incubations prior to t-BuOOH exposure (0 to 250 μM).

In addition, further experiments was performed in order to investigate the effect of 0, 1.75, and 2.5 μM GTG, or 0, 1, 2.5, 5, 7.5 or 10 μM GTG on the susceptibility of EAhy926 cells to t-BuOOH-mediated cytotoxicity. Associated experiments on the effect of all concentrations of GTG (used for t-BuOOH cytotoxicity studies) on selenoenzyme activities have been performed (section 3.2.11).

3.2.12 Assessment of the effect of consecutive sodium selenite and gold thioglucose pre-treatment on susceptibility of EAhy926 cells to oxidative damage resulting from t-BuOOH exposure

To investigate whether the protection of EAhy926 cells from t-BuOOH-mediated cytotoxicity by Se could overcome/compensate for the deleterious effect of GTG pre-incubation, the following approach was employed.

EAhy926 cells were passaged into either Se-deficient medium or medium supplemented with 40 nM sodium selenite, and incubated for 48 hr. After this time, the cells were washed twice with 1 ml EBSS, and received normal unsupplemented medium, or the same medium containing 10 μM GTG for 48 hr. When the incubation was finished, the cells were again washed, and fresh medium containing various concentrations of t-BuOOH (0 – 100 μM) added for a 20 hr incubation. Both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.13.

The same experiment was repeated, with the concentration range of t-BuOOH extended to 250 μM to confirm the effects seen in the first experiment over a more appropriate portion of the toxicity curve.

In the final two experiments, EAhy926 cells received either no pre-incubation (control cells), 40 nM sodium selenite alone, 1 μM GTG alone, 10 μM GTG alone, or 40 nM selenite followed by 1 μM GTG as a pre-incubation before t-BuOOH exposure, as follows. EAhy926 cells were passaged into either Se-deficient medium or medium supplemented with 40 nM selenite, and incubated for 48 hr. After this time, the cells were washed twice with 1 ml EBSS, and received normal unsupplemented medium, or the same medium containing either 1 μM GTG or 10 μM GTG for 48 hr. When the incubation was finished, the cells were again washed, and fresh medium containing various concentrations of t-BuOOH (0 – 250 μM and 0 – 300 μM respectively for the two experiments) added for a 20 hr incubation. Both the medium and cells were harvested and analysed for % LDH retention as described in section 2.3.13.

3.2.13 The ability of sodium selenite to protect against oxidative damage resulting from oxLDL exposure in EAhy926 cells

EAhy926 cells were passaged into 24 well plates and left to grow in Se-deficient medium for 48 hr. The medium was then replaced with fresh unsupplemented medium, or medium supplemented with 40 nM sodium selenite, and the cells left to incubate 48 hr. At this stage the cells were at confluence. After this pre-incubation, all cells were washed twice with 1 ml EBSS, and medium containing either native or oxidised LDL (prepared from the same blood donation, and diluted to a common protein value in medium) was added. This was left to

incubate with the cells for 24 hr. LDH activity was then measured in the medium and cell lysates as described in section 2.3.11, and % LDH retention calculated. Three different LDL preparations were used, the third batch having two paired fractions of both native and oxidised LDL (two isolated fractions native LDL were made from one large pool plasma, which were then oxidised). Total protein was measured in the LDL using the Bradford assay (section 2.3.9) prior to dilution and addition to the cells.

One experiment was performed with oxLDL alone (no native LDL), using sodium selenite (40 nM) and/or GTG (1 μM) pre-incubations (each for 48 hr) to investigate whether GTG would increase the susceptibility of the cells to oxLDL-mediated cytotoxicity, and whether sodium selenite would be able to overcome this deleterious effect.

The endogenous LDL concentration was measured in the FBS used to supplement the culture medium using a method routinely used in clinical biochemistry to measure HDL cholesterol in human serum (see section 2.3.17). The concentration of LDL was measured at 0.404 mmol/L using the equation: LDL cholesterol = Total cholesterol – (HDL cholesterol + 0.16 X Triglycerides). However, this equation is accurate only at levels of >0.5 mmol/L HDL for the assay conditions used, which did not hold true for this measurement. Thus, this offers only a rough approximation. In addition, this assay is set up to measure human lipoproteins, but the measurement here was of bovine, thus introducing further inaccuracy. The amount of LDL in FBS has previously been measured to be 0.76 ± 0.04 mM/L by Saint-Marie *et al.* (1989) (Saint-Marie *et al.*, 1989). Such low values fall in line with what is generally known that serum cholesterol is much lower in animals than in man; not all of this will be LDL as the relative amount of HDL : LDL tends to be higher in animals than in man (personal communication, Prof. R. Riemersma, Dept. Cardiovascular Biology, University of Edinburgh).

3.2.14 The effect of sub-lethal oxLDL concentrations on the expression of TR in EAhy926 cells

Five different batches of LDL were used to investigate the effect of sub-lethal oxLDL on TR expression in EAhy926 cells. Only one was of the same batch as in protection experiment (figures 3.37 c and 3.39 a (c)). LDL was prepared from native LDL isolated from fresh serum by ultracentrifugation, and oxLDL was prepared by oxidation of native LDL by 20 μM $CuCl_2$ (section 2.3.12).

EAhy926 cells were pre-incubated and exposed to LDL as detailed in section 3.2.13. The % LDH activity retention was then measured in the medium (section 2.3.13), to monitor cytotoxicity. The cell lysates for which LDL concentrations had been shown to be non-toxic were frozen at - 80° until RIA for TR (section 2.3.6) was performed. Total protein in the samples was measured by the Bradford assay system (section 2.3.9).

3.2.15 The effect of homocysteine on the expression of selenoproteins in EAhy926 cells

EAhy926 cells were passaged into 75 cm^2 flasks and grown to confluence. The cells then received medium containing 1 mM homocysteine, except 4 flasks which were harvested as controls to serve as the zero time point. Triplicate flasks were incubated with the homocysteine for 4, 10, 24 and 48 hr respectively. At the end of each respective incubation period, the medium was sampled and analysed for LDH activity, as described in section 2.3.13, to check for any cytotoxicity. The cells were then washed twice with 10 ml EBSS, and harvested as detailed in section 3.2.1.

3.2.16 The potential of EAhy926 cells and HUVEC to oxidise native LDL, and the effect of Se supplementation on the oxidation process

EAhy926 cells and HUVEC were passaged into 24-well plates at a seeding density of 5×10^5 cells/cm^2 and 3000 cells/cm^2 respectively, in DMEM or EGM-2 growth medium, respectively. The HUVEC culture medium was changed for medium M199 (supplemented with EGM-2 bulletkit) the following day. The cells were then left to grow in this medium for 48 hr. After the cells had been growing for 48 hr, half of each plate was given medium (M199 in the case of HUVEC, and DMEM for EAhy926 cells) containing 40 nM selenite, while the other wells received their normal, unsupplemented medium. The cells were left to incubate for 48 hr. Following this incubation, the cells were washed twice with 1 ml PBS, and the cells received native LDL (200 μg/ml). Prior to addition to the cells, the total protein content of the LDL was measured using the Bradford protein assay (section 2.3.9), and the LDL was diluted to 200 μg/ml in either serum-free DMEM or serum-free Ham's F-12 for EAhy926 cells, or in serum-free M199 (supplemented with the EGM-2 medium bulletkit, except for the FCS) for the HUVEC. The diluted native LDL was divided into portions, which were either left unsupplemented, or was supplemented with 2.5 μM $CuSO_4$ or 5.0 μM $CuSO_4$ (final concentration). Addition of PBS was used as a control for $CuSO_4$. The incubation with the native LDL was for 24 hr. Native LDL in the wells (± $CuSO_4$) without cells was included for the incubation period as the LDL control. All experimental conditions were arranged in duplicate wells. Normal serum was included in each lipoprotein gel run to serve as a standard.

Following the incubation, the media was removed from the wells, and butylated hydroxytoluene (BHT) and EDTA were added to the sampled media to arrest any further oxidation. BHT and EDTA were added at final concentrations of 25 μM and 100 μM respectively. The samples were then centrifuged at 2000 rpm for 10 min, and put on ice until they were loaded onto a lipoprotein gel (section 2.3.18).

3.2.17 Statistical analysis

One-way analysis of variance (ANOVA) was used to test for significant differences in % LDH activity retained in response to different concentrations of t-BuOOH or oxLDL. In the event that the variation was significant ($p > 0.05$), a Tukey-Kramer multiple comparisons post-test was used to test for the level of significance of differences in % LDH retained in response to t-BuOOH or oxLDL. One-way ANOVA and a Tukey-Kramer multiple comparisons post-test were also used to investigate significant differences between levels of selenoprotein expression and activity in cells cultured in different concentrations of sodium selenite. In the event of large SDs in a particular data set (> 3 SDs from the mean), the data was log transformed prior to ANOVA evaluation.

The different groups of cells (e.g. Se-deficient cells versus Se-supplemented cells) were compared at individual t-BuOOH or oxLDL concentrations using the Student's t-test for unpaired data. The Student's t-test for unpaired data was also used to compare selenoprotein expression between different endothelial cell types, and for the activities of selenoenzymes of cells at different confluence levels.

3.3 RESULTS

All graphs presented are the data from a single experiment, using culture flasks/wells in triplicate, unless stated otherwise.

3.3.1 Intracellular selenoprotein expression in EAhy926 cells and HUVEC

Figure 3.01 demonstrates that the overall pattern of [^{75}Se]-selenoprotein expression in EAhy926 cells resembles that observed in HUVEC with some significant differences in the levels of expression of a few selenoproteins. TR is dominantly expressed to a similar extent in both HUVEC and EAhy926 cells. Figure 3.02 ('a' and 'b') compares the TR activity and concentration of both cell types and confirms that there is no significant difference in the TR expression in these two cell types.

A lower expression of both cyGPX and PHGPX in EAhy926 cells compared to HUVEC is illustrated in figure 3.01. Figure 3.02 confirms this observation, showing that cyGPX activity is 73 % lower in EAhy926 cells (23 + 0.5 U/g protein, mean + SD, n=3) compared to HUVEC (86 + 0.7 U/g protein, mean + SD, n=3) ($p < 0.0001$). Although the PHGPX activity in the EAhy926 cells illustrated a tendency towards being lower than that observed in HUVEC, this difference was not statistically significant (figure 3.02 c).

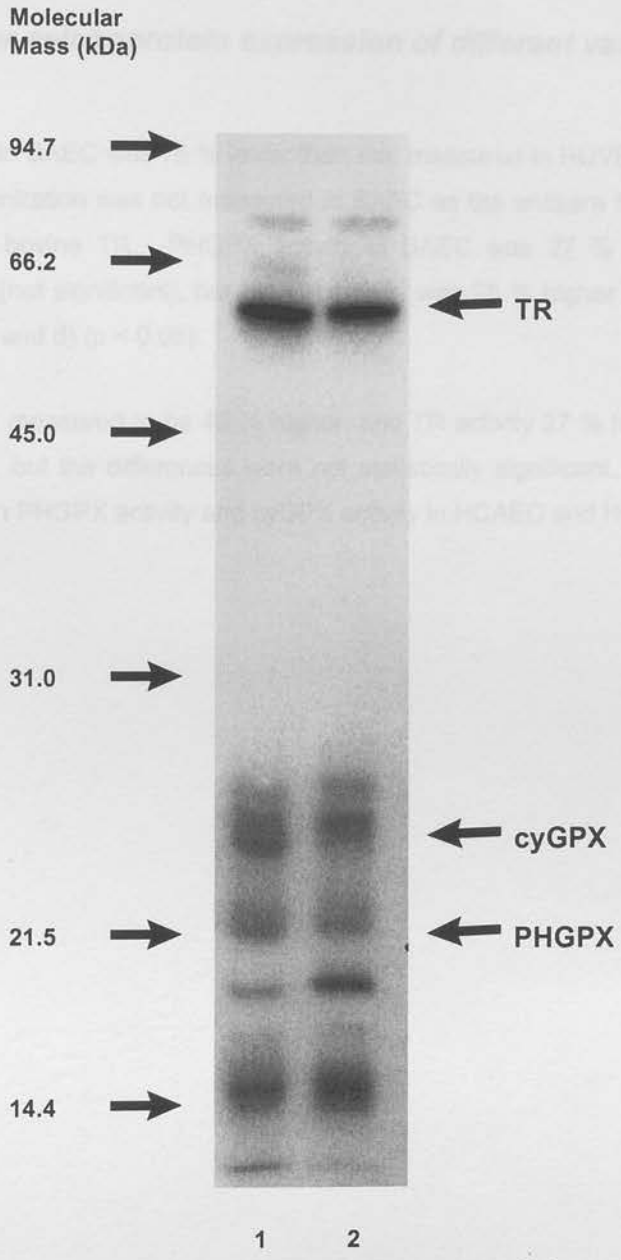


Figure 3.01 Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of human umbilical vein endothelial cells (HUVEC) and EAhy926 cells labelled with [^{75}Se]-selenite (0.02 MBq/ ml) for 48 hr. Lane 1, HUVEC; lane 2, EAhy926 cells. Both lanes were loaded with 25 μg protein. TR, thioredoxin reductase; cyGPX, cytoplasmic glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase.

3.3.2 *Intracellular selenoprotein expression of different vascular endothelial cells*

TR activity measured in BAEC was 76 % lower than that measured in HUVEC (figure 3.02a) ($p < 0.05$). TR concentration was not measured in BAEC as the antisera to human TR did not cross react with bovine TR. PHGPX activity in BAEC was 27 % lower than that measured in HUVEC (not significant), but cyGPX activity was 25 % higher in BAEC than in HUVEC (figure 3.02 c and d) ($p < 0.05$).

TR concentration was measured to be 42 % higher, and TR activity 27 % higher in HCAEC compared to HUVEC, but the differences were not statistically significant. There were no significant difference in PHGPX activity and cyGPX activity in HCAEC and HUVEC.

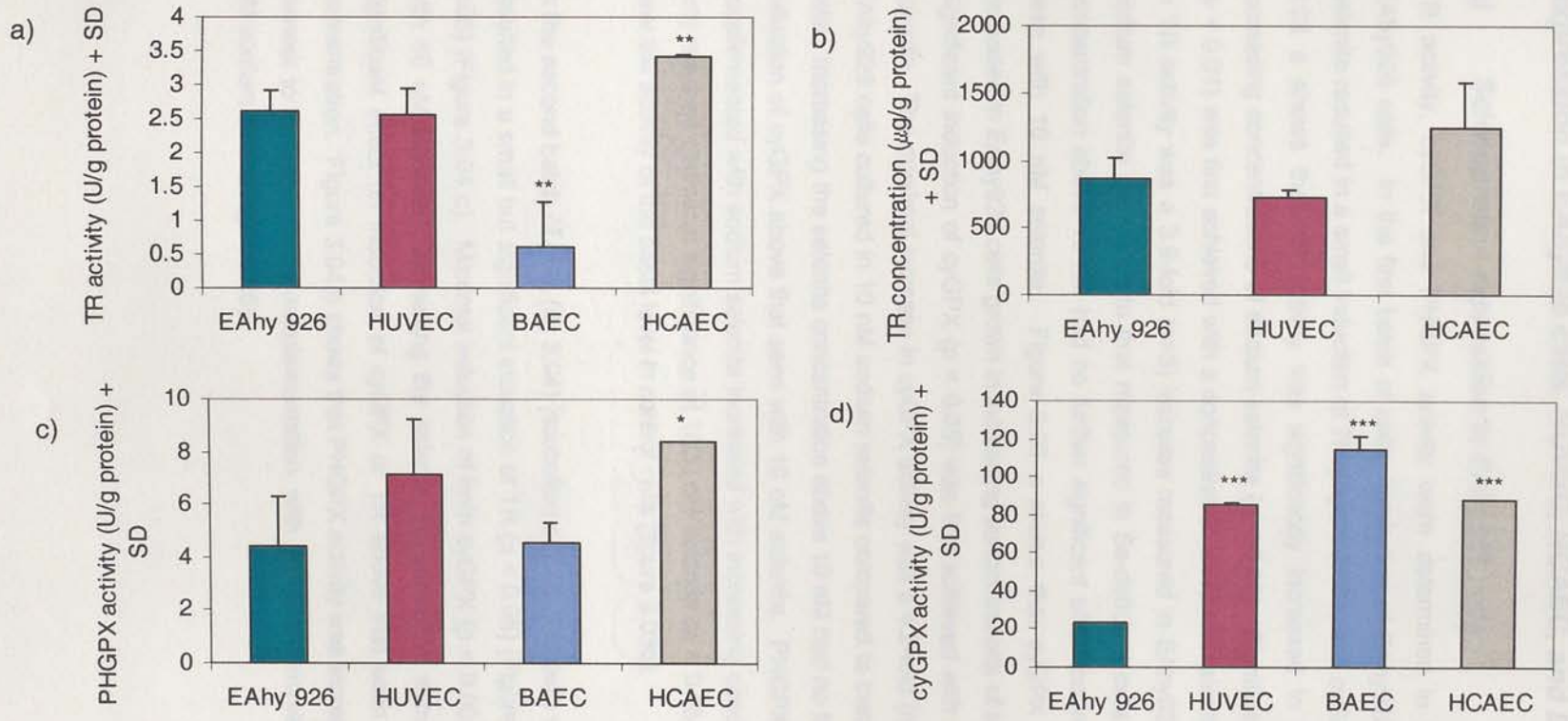


Figure 3.02 Thioredoxin reductase (TR) activity (a) and concentration (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) comparison between EAhy926 cells and human umbilical vein endothelial cells (HUVEC), bovine aortic endothelial cells (BAEC), and human coronary artery endothelial cells (HCAEC). Results shown are the mean of three flasks + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.0001$*** cf. EAhy926 cells. TR expression was not determined in BAEC due to lack of cross-reactivity of the antibody to human TR with the bovine protein. Where SD bars are not visible, they are too small to show on the scale presented.

3.3.3 The effect of sodium selenite supplementation on selenoprotein expression in EAhy926 cells, HUVEC, HCAEC, and BAEC

a) Selenoprotein expression in EAhy926 cells

TR activity, cyGPX and PHGPX activity were determined in two different batches of EAhy926 cells. In the first batch of cells, incubation of EAhy926 cells with 1 nM sodium selenite resulted in a small induction of TR (Figure 3.03a) and cyGPX (Figure 3.03c). Figure 3.03 a shows that TR activity was significantly increased in EAhy926 cells grown in increasing concentrations of sodium selenite ($p < 0.05$). Significant induction of TR activity ($p < 0.01$) was first achieved with a concentration of 10 nM selenite. The maximal increase in TR activity was a 3.9-fold (n=3) increase measured in EAhy926 cells cultured in 10 nM sodium selenite compared to that measured in Se-deficient cells. Increasing the selenite concentration above 10 nM had no further significant effect on induction of TR above that seen with 10 nM selenite. Figure 3.03 c shows that cyGPX activity was significantly increased in EAhy926 cells grown in increasing concentrations of sodium selenite ($p < 0.05$). Significant induction of cyGPX ($p < 0.05$) was first achieved with a concentration of 10 nM selenite. The maximal increase in cyGPX activity was a 4.2-fold (n=3) increase measured in EAhy926 cells cultured in 10 nM sodium selenite compared to that measured in Se-deficient cells. Increasing the selenite concentration above 10 nM had no further significant effect on induction of cyGPX above that seen with 10 nM selenite. PHGPX activity in EAhy926 cells supplemented with sodium selenite increased with increasing concentrations of selenite, but only reached statistical significance at 1000 nM selenite ($p < 0.05$), with a 2.2-fold increase over the activity of the basal level in control cells (figure 3.03b).

In the second batch of cells (fig 3.04) incubation of EAhy926 cells with 1 nM sodium selenite resulted in a small but significant induction of TR ($p < 0.05$) (Figure 3.04 a), and cyGPX ($p < 0.05$) (Figure 3.04 c). Maximal induction of both cyGPX ($p < 0.001$) and TR ($p < 0.01$) was with 40 nM selenite. Increasing the selenite concentration above 40 nM had no further significant effect on induction of cyGPX or TR above that seen with the 40 nM selenite concentration. Figure 3.04 b shows that PHGPX activity was increased in a dose dependent manner to sodium selenite supplementation, with significant increases at 200 nM and 1000 nM sodium selenite ($p < 0.05$).

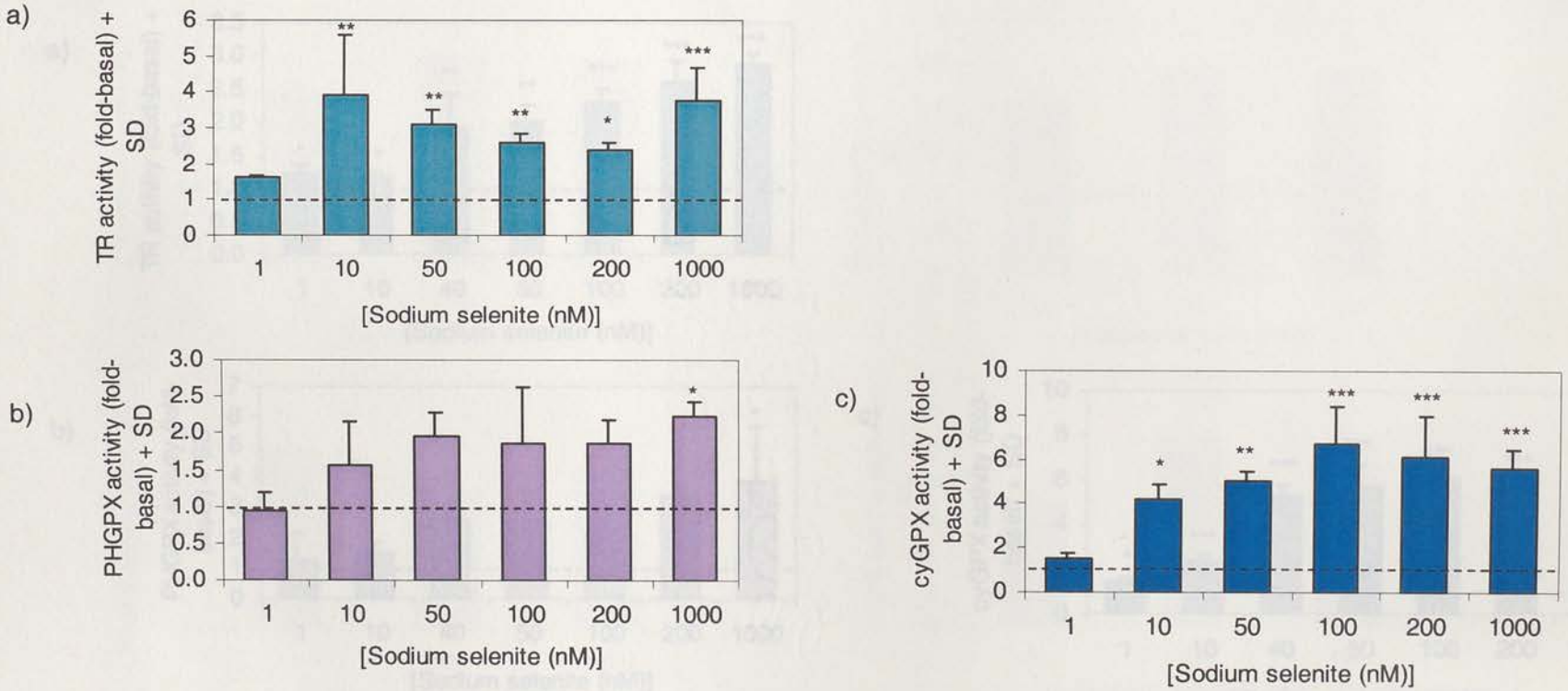


Figure 3.03 Thioredoxin reductase activity (TR) (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in EAhy926 cells supplemented with sodium selenite for 48 hr. Results shown are those of the mean of triplicate flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. Se-deficient control cells.

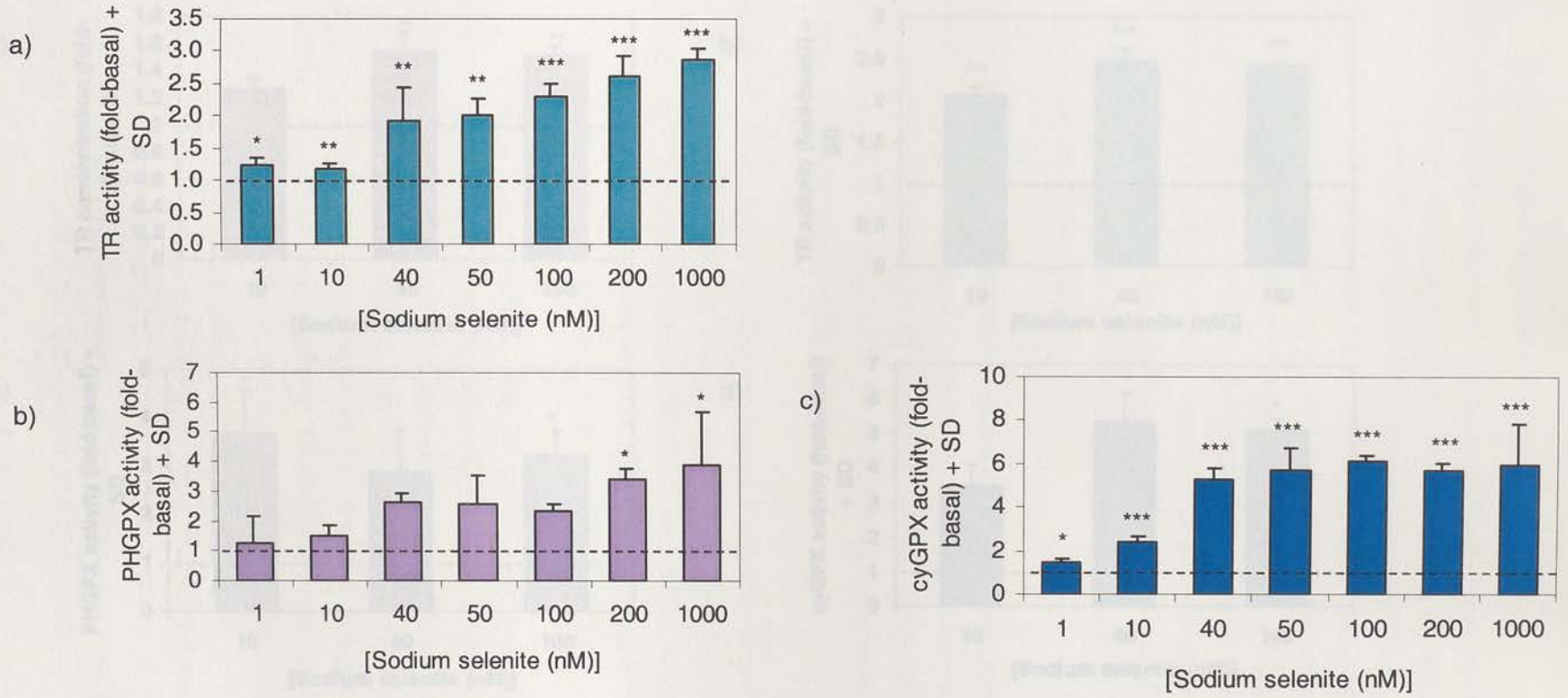


Figure 3.04 Thioredoxin reductase (TR) activity (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in EAhy926 cells supplemented with sodium selenite for 48 hr. Results shown are those of the mean of triplicate flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. Se-deficient control cells.

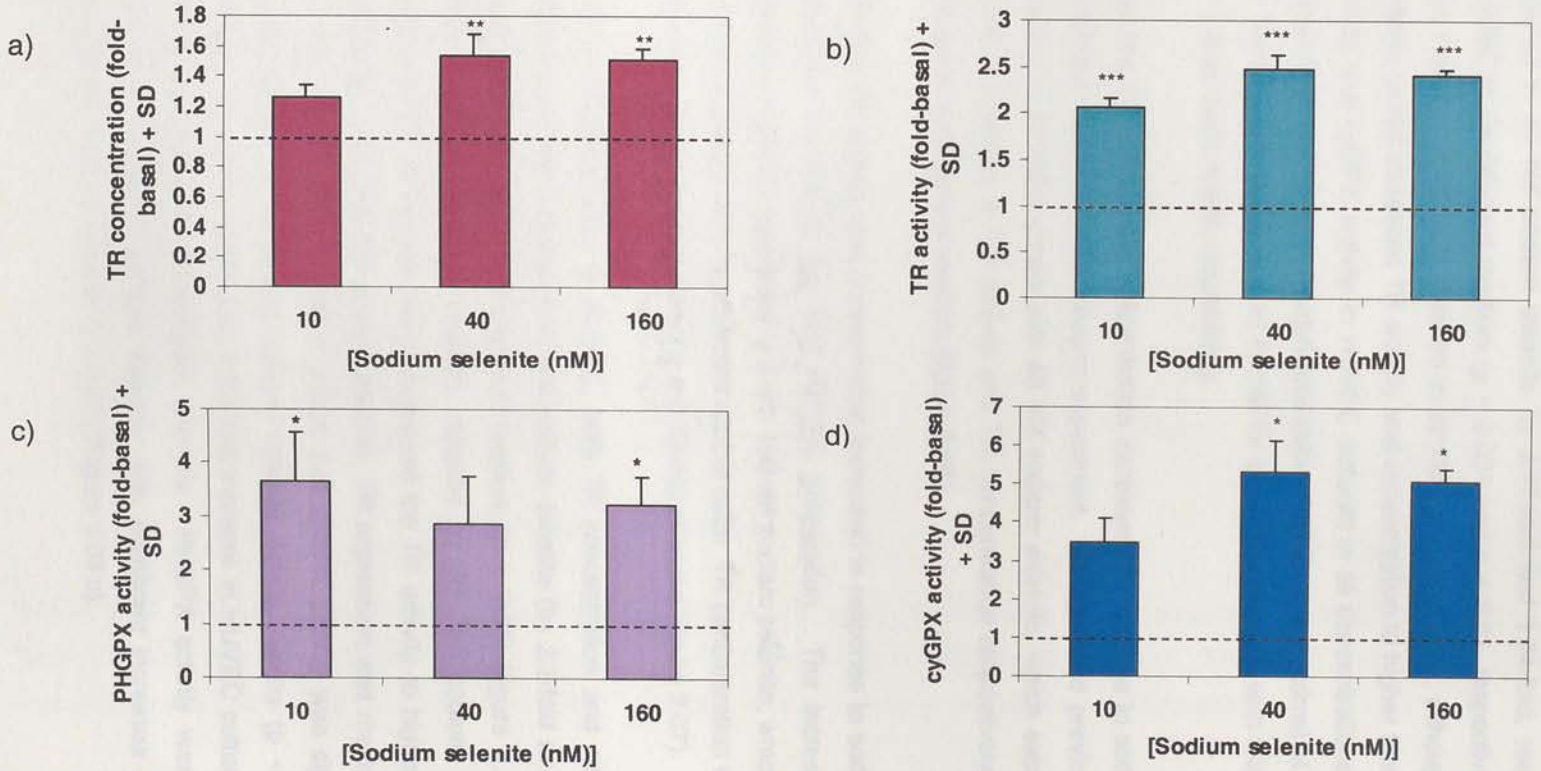


Figure 3.05 Thioredoxin reductase (TR) concentration (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in human umbilical vein endothelial cells (HUVEC) supplemented with sodium selenite for 48 hr. The results are those of the mean of triplicate flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*; $p < 0.01$ **; $p < 0.001$ *** cf. Se-deficient control cells.

b) Selenoprotein expression in HUVEC

TR activity and concentration and cyGPX and PHGPX activity was determined in HUVEC cultured in different concentrations of sodium selenite (0, 10, 40, 160 nM). Figure 3.05 a and b show that both TR activity and concentration were significantly increased in HUVEC cultured in 40 nM sodium selenite by 2.47-fold and 1.54-fold, respectively, compared to HUVEC in Se-deficient medium ($p < 0.001$ and $p < 0.01$, respectively). Expression of TR was maximal in HUVEC cultured in 40 nM sodium selenite, although all concentrations of selenite tested increased TR activity and concentration to higher than basal levels. PHGPX activity and cyGPX activity in HUVEC cultured in all concentrations of selenite tested was higher than the levels in Se-deficient cells. Activity was maximal at 10 nM sodium selenite for PHGPX ($p < 0.05$), and at 40 nM for cyGPX ($p < 0.05$), with fold increases of 3.65 and 5.31 over basal levels, respectively.

Both the TR activity and concentration increased in response to sodium selenite in a dose-dependent manner in the second experiment. As observed previously, the increase was maximal in HUVEC grown with 40 nM sodium selenite, which expressed a 3.96-fold and 2.03-fold increase in TR activity and TR concentration respectively over cells which were cultured in Se-deficient medium (figure 3.06).

Both the TR activity and concentration increased in response to sodium selenite in a dose-dependent manner in the third HUVEC preparation. The increase in TR activity was maximal in HUVEC supplemented with 160 nM sodium selenite, which expressed a 6.39-fold increase in activity over Se-deficient control cells. TR concentration was maximal in HUVEC cultured in 40 nM sodium selenite, a 3.70-fold increase (figure 3.07).

In a fourth preparation of HUVEC, both TR concentration and activity were significantly increased in cells cultured in 40 nM sodium selenite (by 2.2-fold and 3.0-fold, respectively) compared to HUVEC in Se-deficient medium ($p < 0.05$) (figure 3.08 a and b). The TR activity was maximal in HUVEC cultured in 40 nM sodium selenite, although all concentrations of selenite tested increased the TR activity to higher than that seen in the basal state. At 1000 nM sodium selenite, TR expression was maximally increased by 2.9-fold over basal. Figure 3.08 d shows that cyGPX activity was significantly increased in HUVEC grown in increasing sodium selenite concentrations ($p < 0.01$). The maximal increase in cyGPX activity was a 5.4-fold increase in HUVEC cultured in 1000 nM sodium selenite compared to Se-deficient controls. PHGPX activity was increased in a dose dependent manner to sodium selenite, with significant increases at 200 nM ($p < 0.05$) and1000 nM sodium selenite ($p < 0.01$) (Figure 3.08 c).

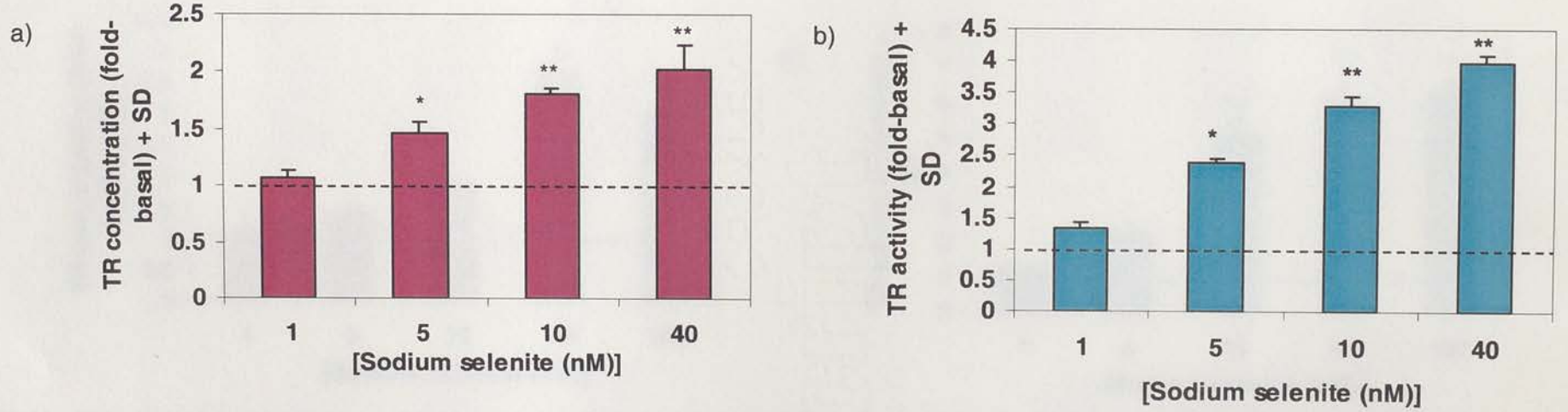


Figure 3.06 Thioredoxin reductase (TR) concentration (a) and activity (b) in human umbilical vein endothelial cells (HUVEC) supplemented with sodium selenite for 48 hr. Results shown are those of the mean of triplicate flasks + SD. The basal level of activity and concentration is indicated by the dashed line. $p < 0.01$*; $p < 0.001$ ** cf. basal level of activity in Se-deficient control cells.

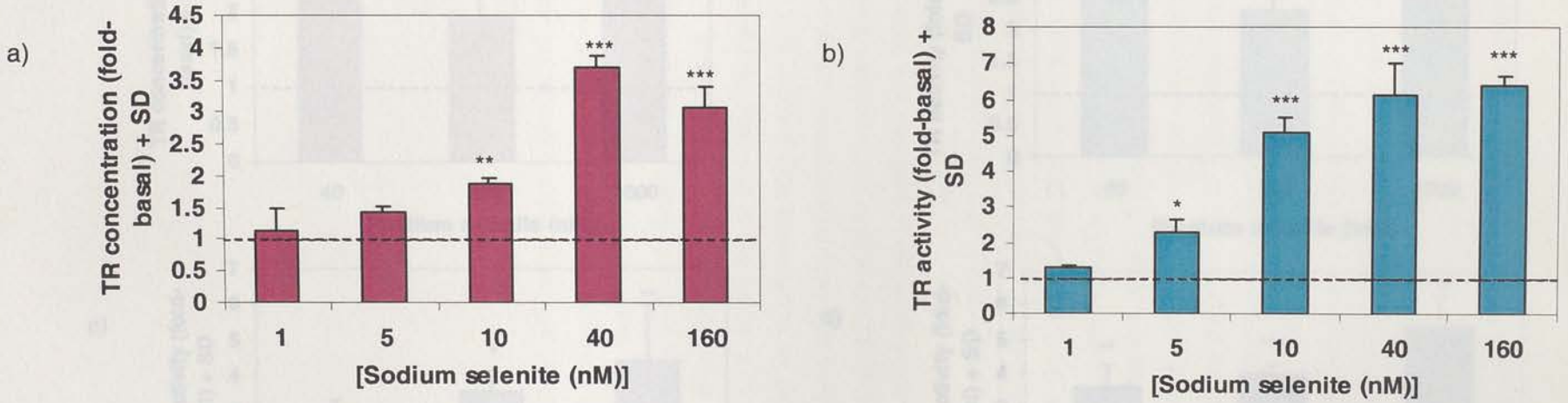


Figure 3.07 Thioredoxin reductase (TR) concentration (a) and activity (b) in human umbilical vein endothelial cells (HUVEC). Results shown are those of the mean of triplicate flasks + SD. The basal level of activity and concentration is indicated by the dashed line. $p < 0.05$ *, $p < 0.01$**; $p < 0.001$ *** cf. basal level of activity in Se-deficient control cells.

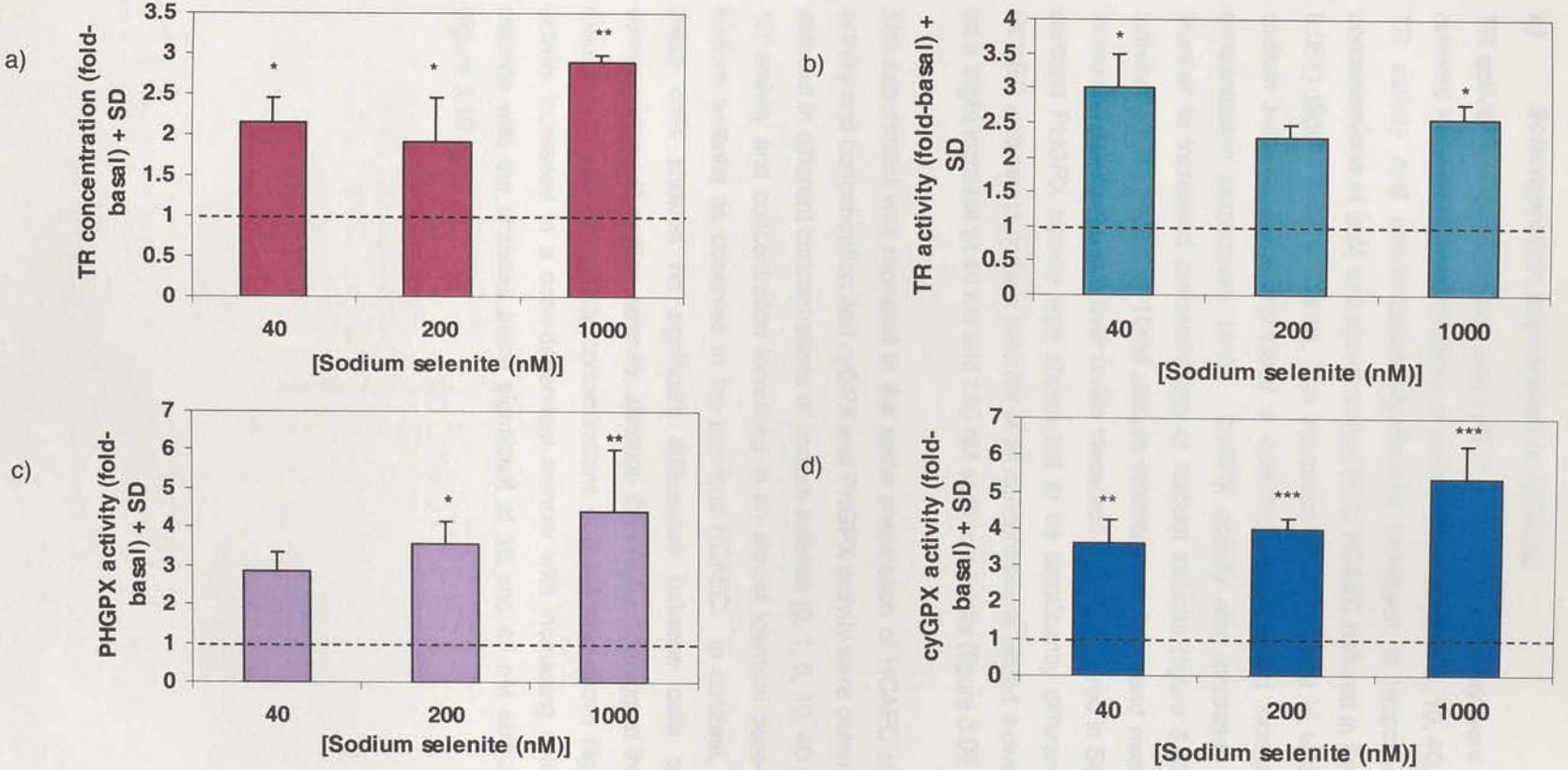


Figure 3.08 Thioredoxin reductase (TR) concentration (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in human umbilical vein endothelial cells (HUVEC). Results shown are those of the mean of triplicate flasks + SD. The basal levels are indicated by the dashed line. $p < 0.05$ *, $p < 0.01$**; $p < 0.001$ *** cf. basal level in Se-deficient control cells.

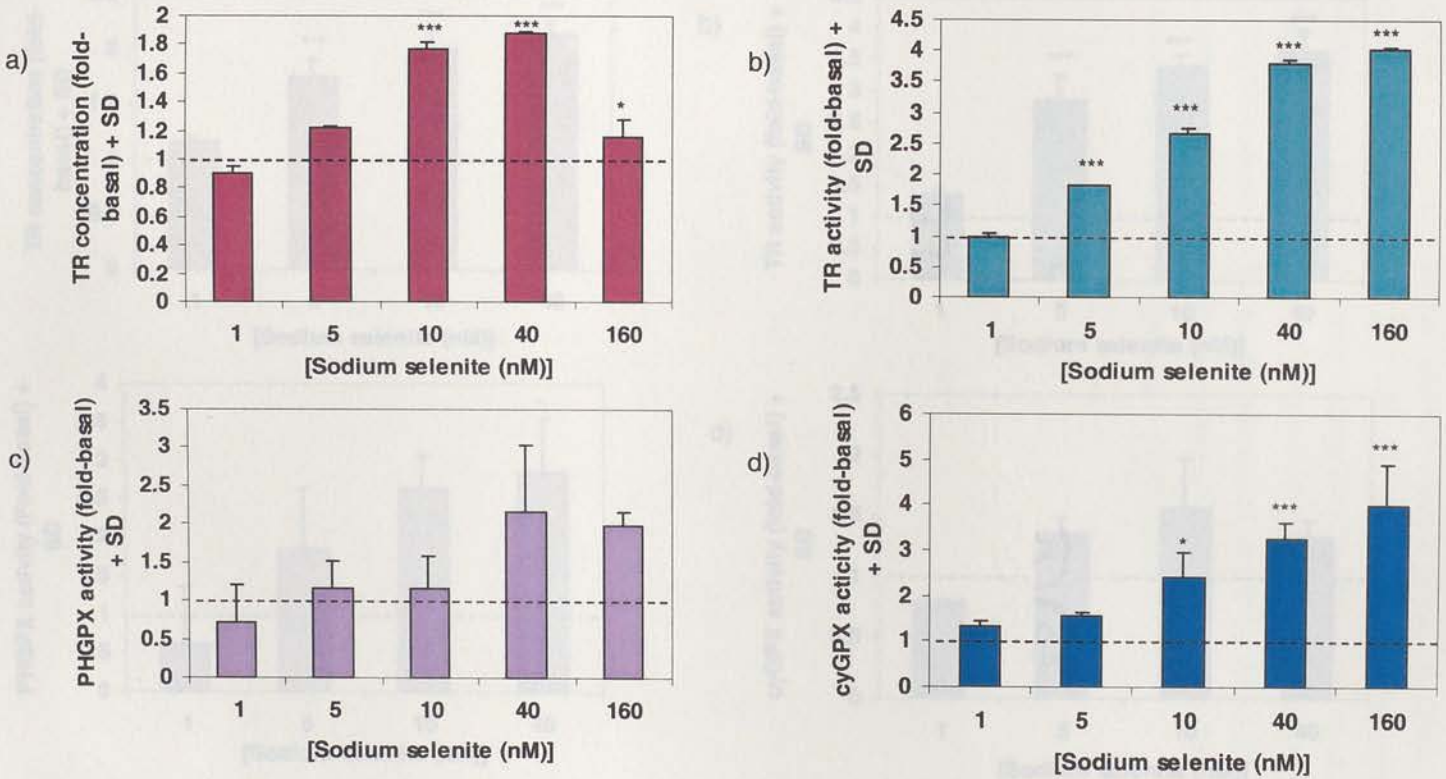


Figure 3.09 Thioredoxin reductase (TR) concentration (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in human coronary artery endothelial cells (HCAEC). Results shown are those of the mean of triplicate flasks + SD. Basal levels are indicated by the dashed line. $p < 0.05$ *, $p < 0.01$**; $p < 0.001$ *** cf. basal level of activity in Se-deficient control cells.

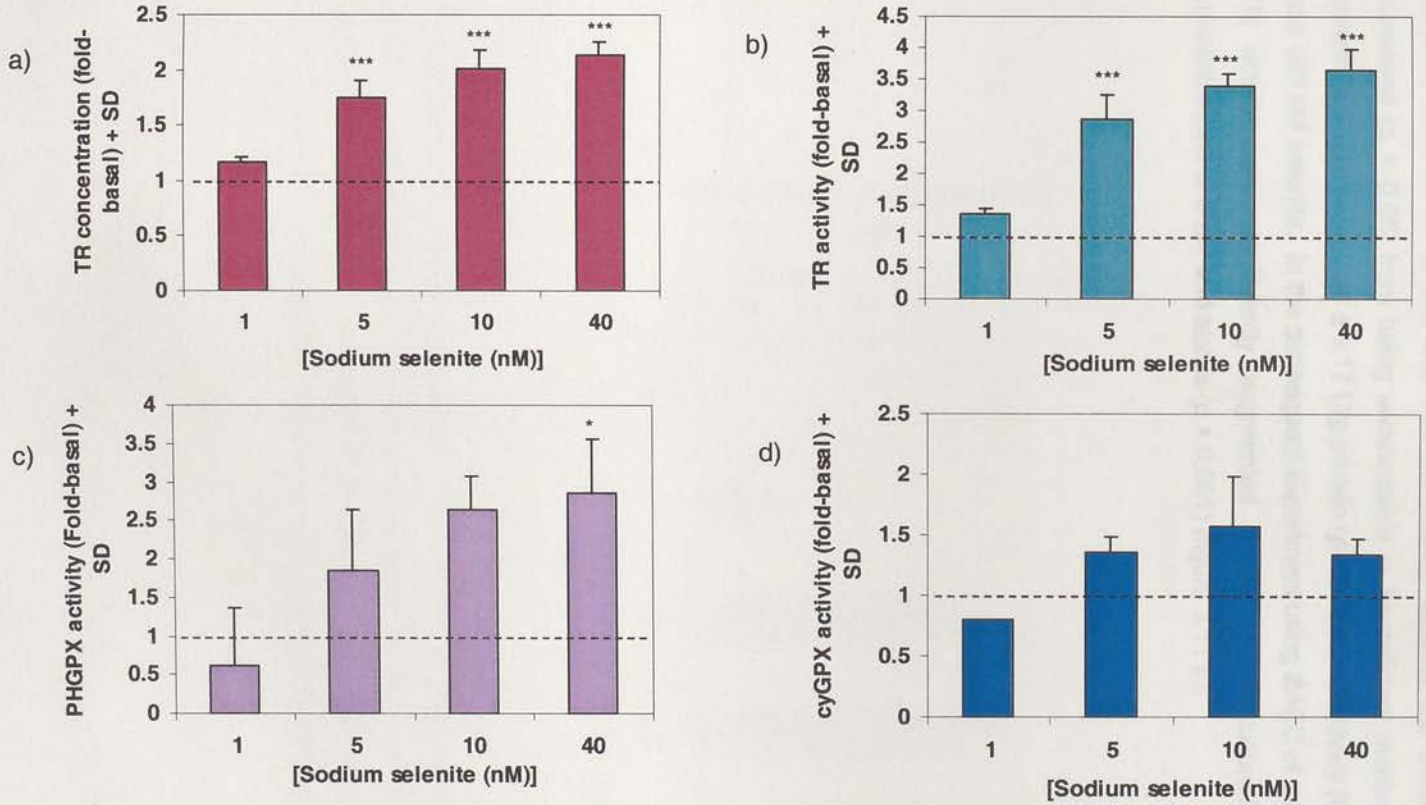


Figure 3.10 Thioredoxin reductase (TR) concentration (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in human coronary artery endothelial cells (HCAEC). Results shown are those of the mean of three flasks + SD. Basal levels are indicated by the dashed line. $p < 0.05$ *, $p < 0.001$ *** cf. basal level in Se-deficient control cells.

d) TR activity in BAEC

TR activity was measured in response to increasing concentrations of sodium selenite in two different passages of BAEC. Figure 3.11 shows the changes in TR activity in BAEC in response to increasing sodium selenite concentrations. In BAEC, of the same passage number, cultured in 40 nM and 160 nM sodium selenite the activity of TR significantly increased ($p < 0.05$) from being undetectable in Se-deficient medium to 0.59 ± 0.12 U/g protein ($p < 0.01$) and 0.48 ± 0.17 U/g protein ($p < 0.05$) respectively (figure 3.11 a) at 40 nM and 160 nM selenite. In the subsequent experiment using BAEC of a later passage number, TR activity was significantly augmented in the BAEC cultured in sodium selenite concentrations of 5 nM and above ($p < 0.001$) (figure 3.11 b).

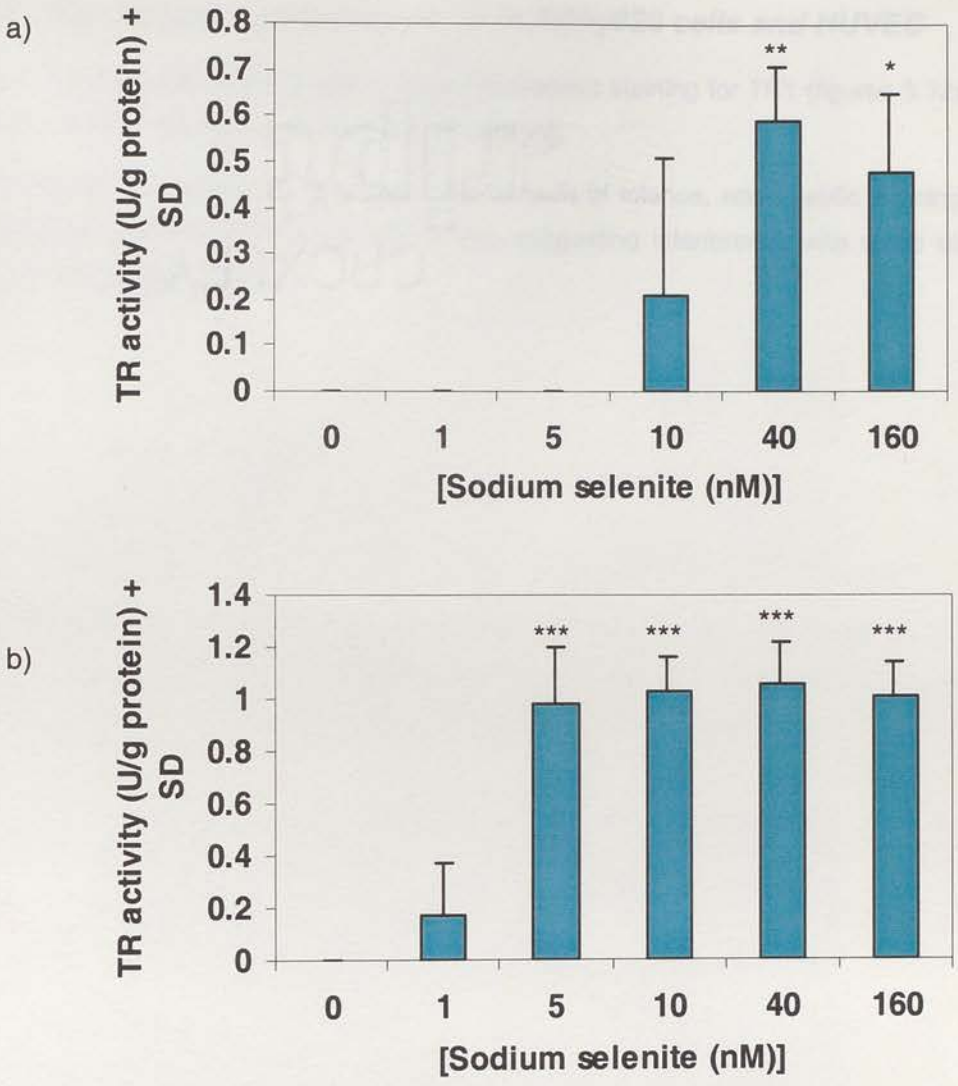


Figure 3.11 Thioredoxin reductase (TR) activity in bovine aortic endothelial cells (BAEC). BAEC of the same preparation were of consecutive passage number for experiments (a) and (b). Results shown are those of the mean of triplicate flasks + SD. $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** cf. basal level of activity in Se-deficient control cells.

3.3.4 The cellular localisation of TR in EAhy926 cells and HUVEC

HUVEC and EAhy926 cells appear to have cytoplasmic staining for TR1 (figures 3.12a and 3.13a). No other cellular locations displayed staining.

The EAhy926 cells displayed a considerable amount of intense, non-specific staining with non-immune serum (figures 3.12b and 3.13c), suggesting interference with some cellular factors in the culture system.

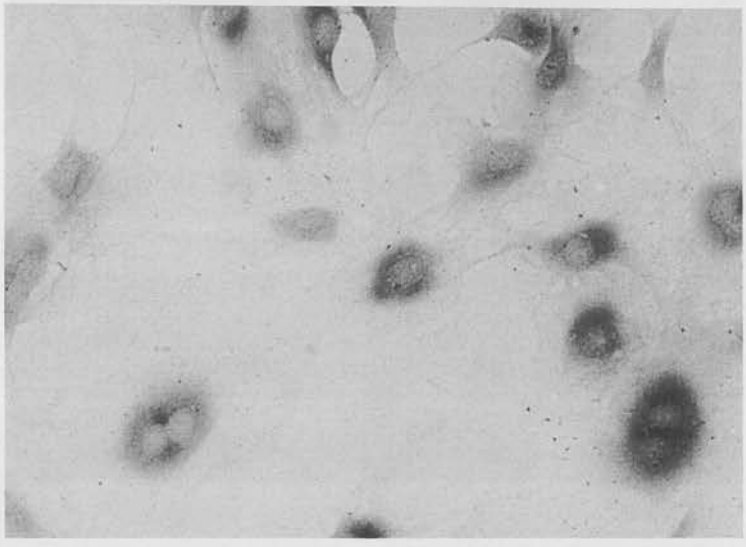


Figure 3.12a Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of human umbilical vein endothelial cells (HUVEC). x 100 magnification. Both the primary and secondary antibodies were present.

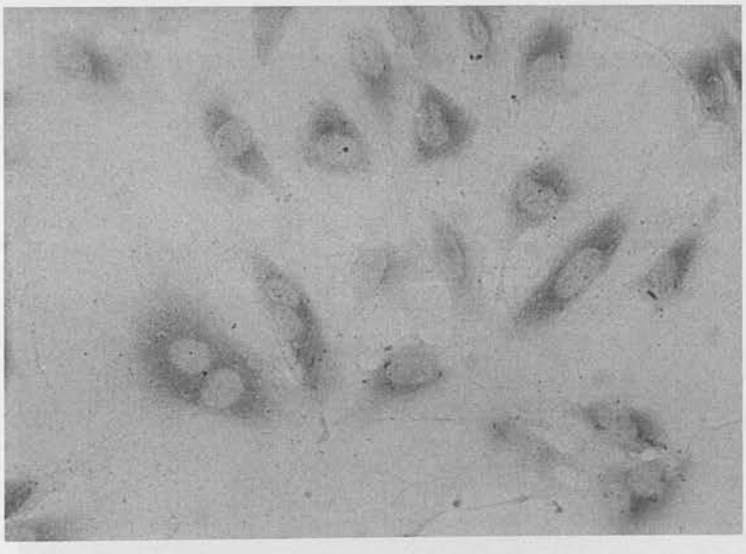


Figure 3.12b Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of human umbilical vein endothelial cells (HUVEC). x 100 magnification. Non-reactive serum was applied as a control.

Figure 3.13a Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of EAhy926 cells. x 100 magnification. Both the primary and secondary antibodies were present.

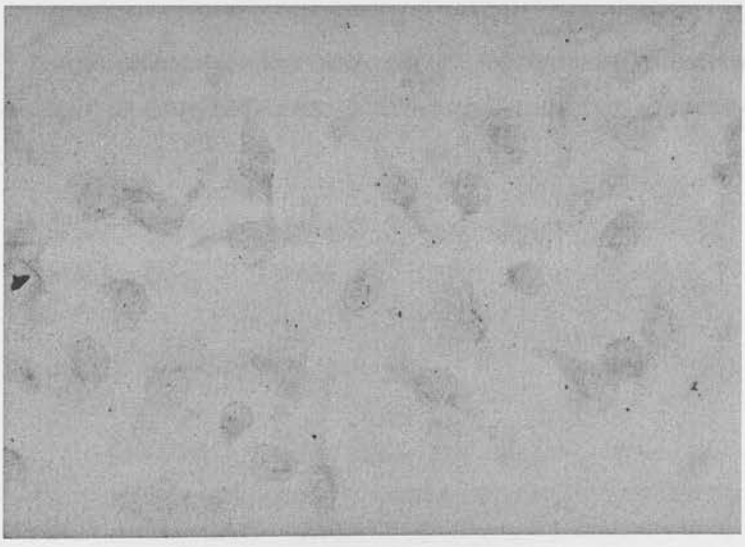


Figure 3.13b Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of EAhy926 cells. x 100 magnification. For the immunohistochemistry here the secondary antibody was present, but without the primary antibody.

Figure 3.13c Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of EAhy926 cells. x 100 magnification. Non-reactive serum was applied as a control.

3.3.5 The effect of different doses of t-BuOOH on LDH activity in EAhy926 cells cultured in selenium-deficient medium

Cell damage of EAhy926 cells was increased in a dose-dependent manner in response to treatment with t-BuOOH (figure 3.14). The cytotoxic effect was initially observed at 175 μM (44.5 ± 3.70 % LDH retention; mean ± SD, n=3). A concentration of 200 μM t-BuOOH further decreased LDH retention to 16.4 ± 0.1 %. Concentrations exceeding 250 μM t-BuOOH gave >95% cell damage such that no LDH retention could be measured in the cells. The inter-experiment reproducibility of cell damage varied greatly during the initial series of experiments; a concentration of 100 μM t-BuOOH yielded LDH retentions of 91.6 ± 1.2 %, 3.5 ± 3.6 %, 83.0 ± 2.7 %, and 97.4 ± 0.5 % respectively in separate experiments (data not shown). This led to an investigation of the effect of cellular confluence level on the susceptibility of EAhy926 cells to oxidative damage by t-BuOOH (section 3.3.6). A cell density plating protocol was employed following these investigations to decrease the variation in the response of cells due to confluence. The EAhy926 cells were passaged at a cell density of 5×10^5 cells/cm^2, which took 4 days to reach confluence.

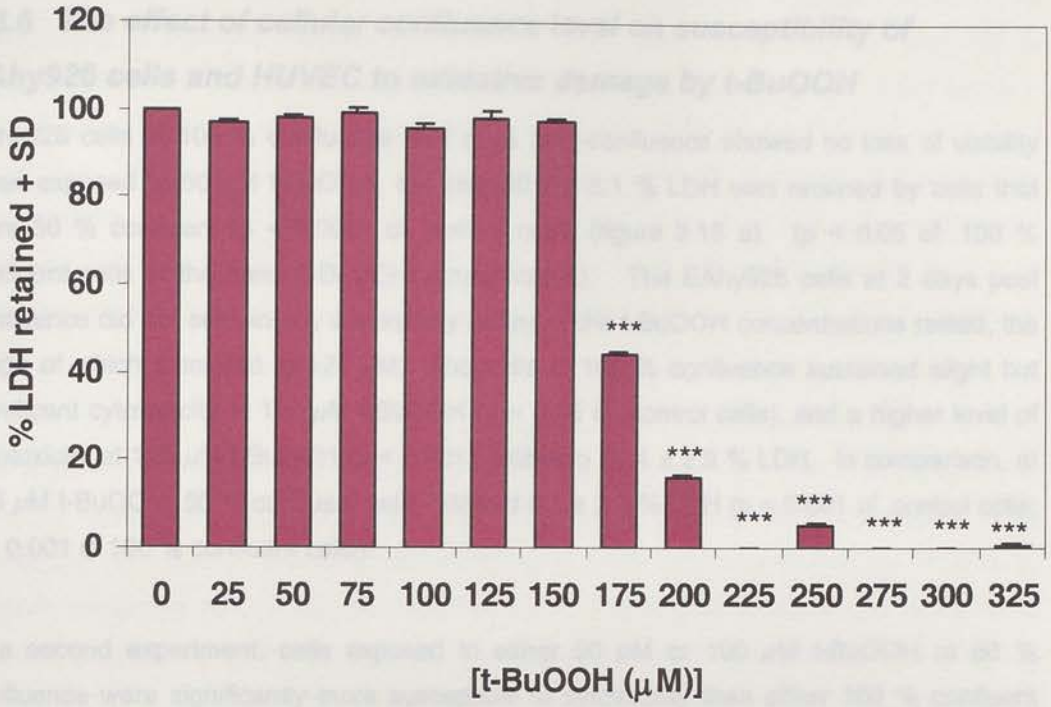


Figure 3.14 The effect of increasing concentrations of tert-butyl hydroperoxide (t-BuOOH) on % LDH retained in EAhy926 cells after 20 hr exposure. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of t-BuOOH (0 to 325 μM, in 25 μM increments) when confluent. Results shown are mean + SD of triplicate wells. $p < 0.01$ **; $p < 0.001$ *** cf. control cells.

3.3.6 The effect of cellular confluence level on susceptibility of EAhy926 cells and HUVEC to oxidative damage by t-BuOOH

EAhy926 cells at 100 % confluence or 2 days post-confluence showed no loss of viability when exposed to 50 μM t-BuOOH, but only 30.6 ± 8.1 % LDH was retained by cells that were 50 % confluent ($p < 0.0001$ cf. control cells) (figure 3.15 a). ($p < 0.05$ cf. 100 % confluent cells at the same t-BuOOH concentration). The EAhy926 cells at 2 days post confluence did not sustain any cytotoxicity at any of the t-BuOOH concentrations tested, the range of which extended to 125 μM. The cells at 100 % confluence sustained slight but significant cytotoxicity at 100 μM t-BuOOH ($p < 0.05$ cf. control cells), and a higher level of cytotoxicity at 125 μM t-BuOOH ($p < 0.001$), retaining 84.4 ± 2.9 % LDH. In comparison, at 125 μM t-BuOOH, 50 % confluent cells retained 9.3 ± 2.3 % LDH ($p < 0.001$ cf. control cells; $p < 0.001$ cf 100 % confluent cells).

In a second experiment, cells exposed to either 50 μM or 100 μM t-BuOOH at 50 % confluence were significantly more susceptible to cytotoxicity than either 100 % confluent cells ($p < 0.01$) or 2 day post-confluent cells ($p < 0.01$) (figure 3.15 b). At a concentration of 150 μM t-BuOOH, 50 % confluent cells were more susceptible to cytotoxicity than were 100 % confluent cells or 2 day post-confluent cells ($p < 0.0001$). At a concentration of 200 μM t-BuOOH, the cytotoxicity to 50 % and 100 % confluent cells was not significantly different, but 2 day post-confluent cells were significantly less susceptible to damage than were 50 % or 100 % confluent cells ($p < 0.05$). This trend of the 2 day post-confluent cells showing less susceptibility to damage was repeated for all the remaining concentrations of t-BuOOH tested ($p < 0.05$).

The time between passage and cytotoxic insult was also found to affect the level of cytotoxicity sustained by EAhy926 cells from t-BuOOH. The cells were seeded at differing densities upon sub-culturing such that both groups of cells achieved full confluence upon cytotoxic insult. The cells left to grow for 2 days between passage and t-BuOOH exposure were significantly more susceptible to cytotoxic damage by t-BuOOH than were the cells left for 4 days between passage and t-BuOOH exposure ($p < 0.001$) (figure 3.16). EAhy926 cells left to grow for 4 days between sub-culture and t-BuOOH exposure did not experience any significant decrease in viability at any of the t-BuOOH concentrations tested; the cells left to grow for 2 days between sub-culture and t-BuOOH exposure sustained significant damage at t-BuOOH concentrations of 150 μM and above ($p < 0.001$).

HUVEC exposed to t-BuOOH at different confluence levels also showed differences in susceptibility to cytotoxic damage (figure 3.17). 1 day pre-confluent HUVEC and 100 % confluent HUVEC first sustained cytotoxicity from 55 μM t-BuOOH ($p < 0.001$), but at this t-BuOOH concentration 2 day post-confluent HUVEC showed no susceptibility to cytotoxicity ($p < 0.05$ cf. 100 % confluent cells). 2 day post-confluent HUVEC first showed susceptibility to cytotoxicity at 70 μM t-BuOOH ($p < 0.01$), retaining 65.5 ± 13.6 % LDH, whilst pre-confluent cells and 100 % confluent cells retained 24.6 ± 5.8 % and 23.4 ± 8.3 % of their LDH respectively ($p < 0.001$) ($p < 0.05$, 2 day post-confluent cells versus 100 % confluent cells).

The level of FCS in the culture medium also influenced the cytotoxicity of t-BuOOH to EAhy926 cells. Cells cultured in medium containing 5 % FCS were significantly more susceptible to t-BuOOH-induced cytotoxicity ($p < 0.05$) than corresponding cultures in medium containing 10 % FCS (data not shown). The toxicity of t-BuOOH was found to be inversely proportional to the concentration of FCS in the medium in which the t-BuOOH is dissolved prior to addition to the cells. The % FCS was kept constant at 10 % for EAhy926 cells in culture for all experiments described.

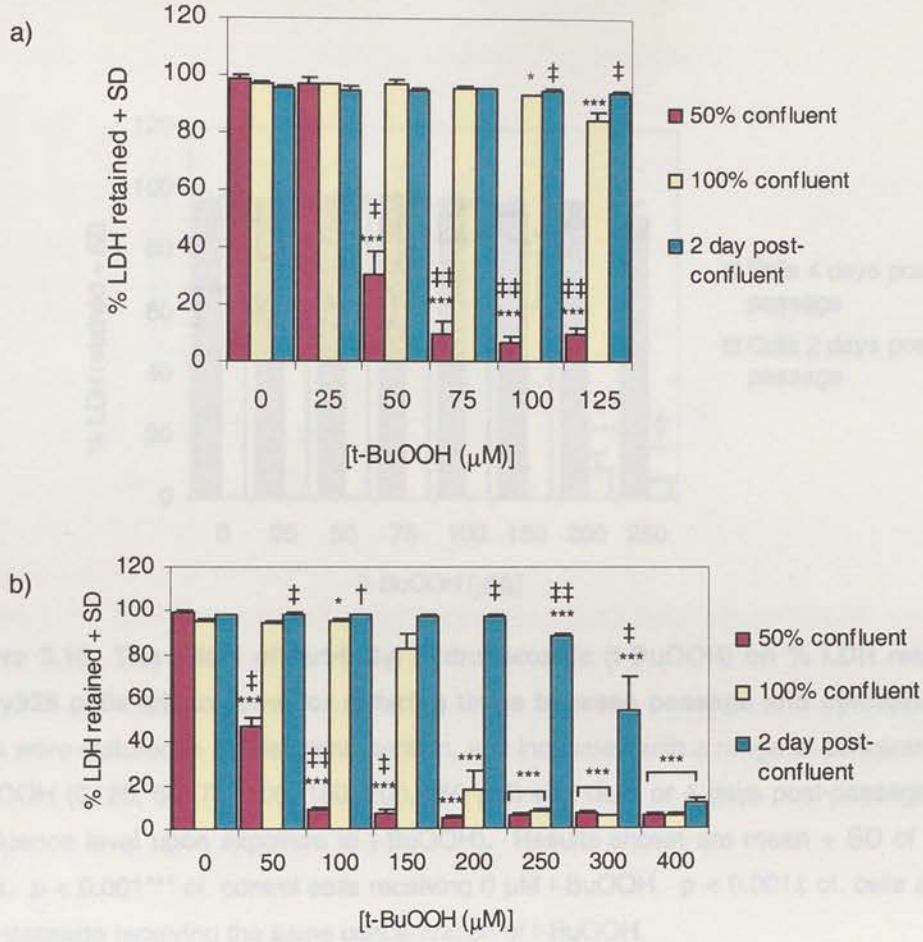


Figure 3.15 The effect of tert-butyl hydroperoxide (t-BuOOH) on % LDH retained in EAhy926 cells grown to differing confluence levels. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of t-BuOOH at 50 % confluence, 100 % confluence, or at 2 days post-confluence. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.001$*** cf. control cells receiving 0 μM t-BuOOH. (a) and (b) are two different experiments. $p < 0.05$†, $p < 0.01$‡, $p < 0.001$‡‡ cf. 100 % confluent cells receiving the same t-BuOOH concentration.

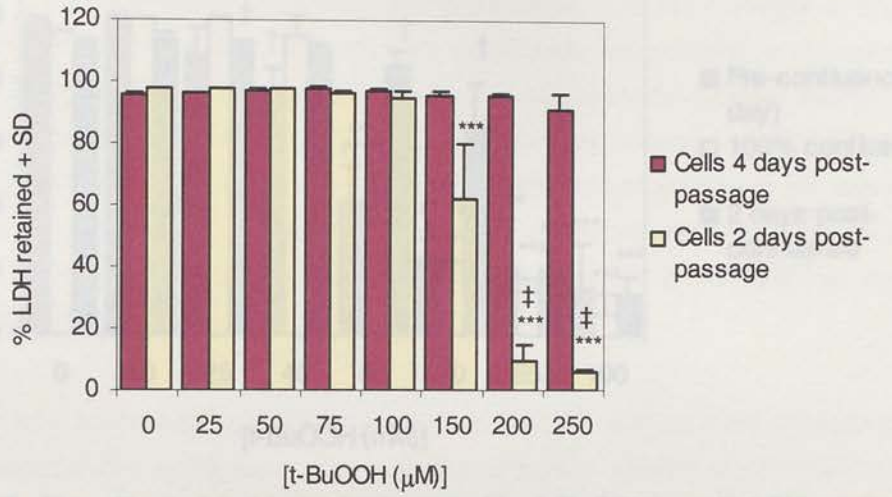


Figure 3.16 The effect of tert-butyl hydroperoxide (t-BuOOH) on % LDH retained in EAhy926 cells left to grow for differing times between passage and cytotoxic insult. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of t-BuOOH (0, 25, 50, 75, 100, 150, 200, 250 μM) at 2 days or 4 days post-passage (similar confluence level upon exposure to t-BuOOH). Results shown are mean + SD of triplicate wells. $p < 0.001$*** cf. control cells receiving 0 μM t-BuOOH. $p < 0.001$‡ cf. cells at 4 days post-passage receiving the same concentration of t-BuOOH.

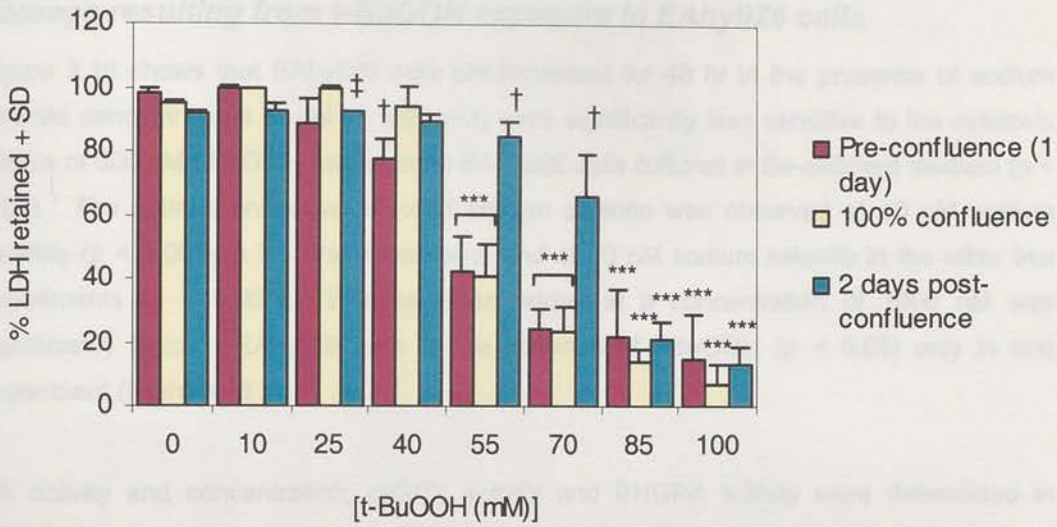


Figure 3.17 The effect of tert-butyl hydroperoxide (t-BuOOH) on % LDH retained in HUVEC grown to differing confluence levels. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of t-BuOOH (0, 10, 25, 40, 55, 70, 85, 100 μM) at 1 day pre-confluence, 100 % confluence, or at 2 days post-confluence. Results shown are mean + SD of triplicate wells. $p < 0.01$**; $p < 0.001$*** cf. control cells. $p < 0.05$†, $p < 0.01$‡ cf. cells at 100 % confluence receiving the same t-BuOOH concentration.

3.3.7 The ability of sodium selenite to protect against oxidative damage resulting from t-BuOOH exposure in EAhy926 cells

Figure 3.18 shows that EAhy926 cells pre-incubated for 48 hr in the presence of sodium selenite concentrations (1 nM to 1000 nM) were significantly less sensitive to the cytotoxic effects of 300 μM t-BuOOH compared to EAhy926 cells cultured in Se-deficient medium ($p < 0.05$). The optimal protective effect of sodium selenite was observed at 40 nM sodium selenite ($p < 0.001$) in the first experiment, and at 10 nM sodium selenite in the other two experiments ($p < 0.001$). Selenite when added at a concentration of 1000 nM was significantly toxic to EAhy926 cells in the absence of t-BuOOH ($p < 0.05$) only in one experiment (figure 3.18 b).

TR activity and concentration, cyGPX activity and PHGPX activity were determined in EAhy926 cells cultured in the same concentrations of sodium selenite as described above for the protection experiments. Figures 3.03 a and c show that both TR activity and cyGPX activity were significantly increased in EAhy926 cells cultured in the presence of 10 nM sodium selenite compared to EAhy926 cells cultured in Se-deficient medium ($p < 0.01$; $p < 0.05$). The TR and cyGPX activities were maximal in EAhy926 cells cultured in 10nM sodium selenite (first experiment) and 40 nM sodium selenite (second experiment), although all the concentrations of sodium selenite tested increased TR activity above that seen in the basal state.

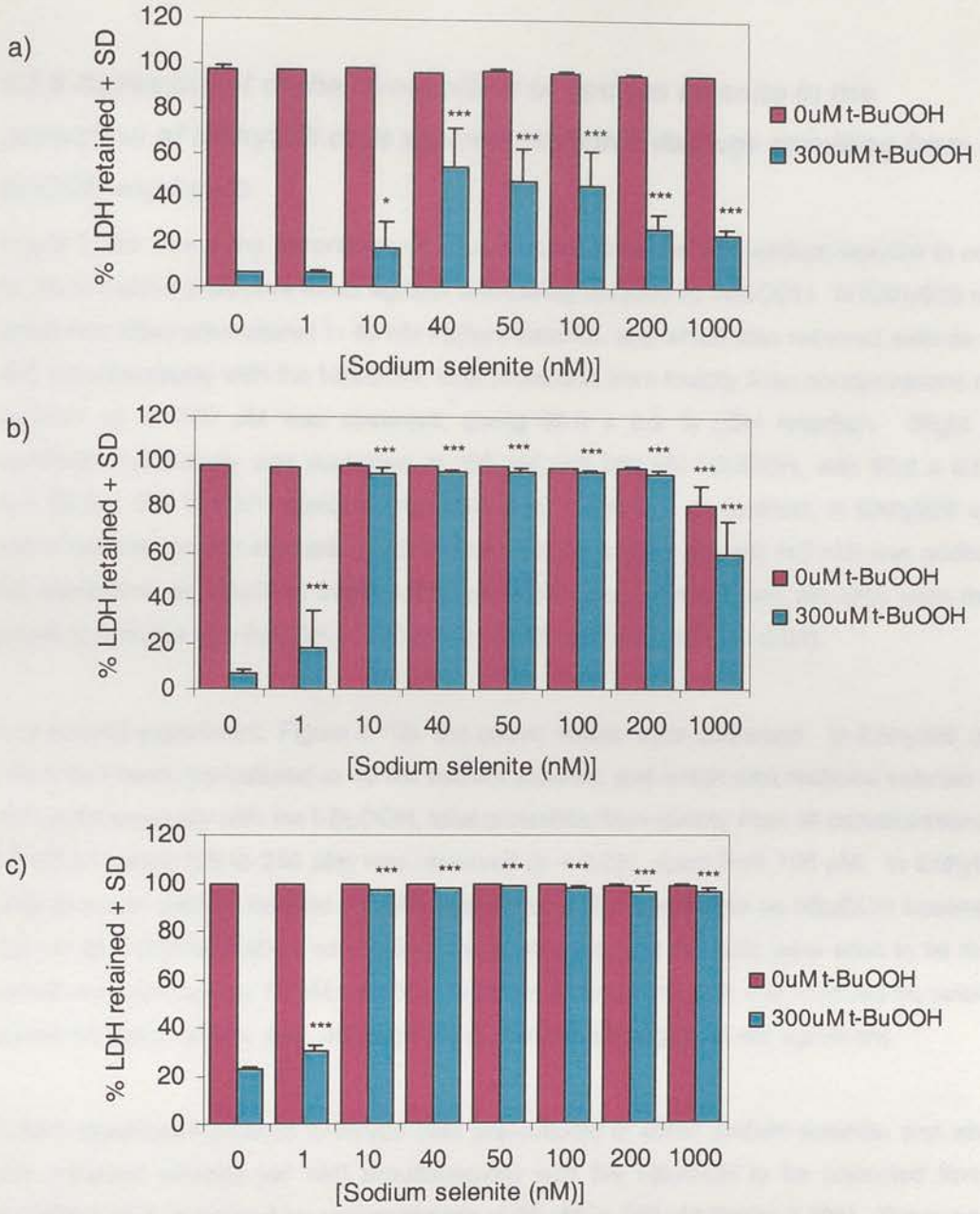


Figure 3.18 The effect of sodium selenite pre-incubation on the susceptibility of EAhy926 cells to tert-butylhydroperoxide (t-BuOOH)-induced cell damage. EAhy926 cells were incubated in Se-deficient medium supplemented with different concentrations of sodium selenite (0, 1, 10, 40, 50, 100, 200, 1000 nM) for 48 hr. Following the pre-incubation the EAhy926 cells were exposed to t-BuOOH (0 or 300 μM). After 20 hr cell viability was assessed by determining the % LDH activity retained. Results shown are the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. Se-deficient control cells. Graphs (a) – (c) are each a separate experiment.

3.3.8 Assessment of the direct effect of sodium selenite in the protection of EAhy926 cells against oxidative damage resulting from t-BuOOH exposure

Figure 3.19a shows the importance of a pre-incubation period with sodium selenite in order for Se to exert a protective effect against cytotoxicity induced by t-BuOOH. In EAhy926 cells which had been pre-cultured in 40 nM sodium selenite, and which also received selenite (40 nM) simultaneously with the t-BuOOH, total protection from toxicity from concentrations of t-BuOOH up to 150 μM was observed, giving 98.0 ± 0.2 % LDH retention. Slight but significant cytotoxicity was sustained at 200 μM and 250 μM t-BuOOH, with 95.8 ± 0.6 % and 94.5 ± 0.6 % LDH retention respectively ($p < 0.001$). In contrast, in EAhy926 cells which received no pre-incubation with selenite but the sodium selenite (40 nM) was added at the same time as t-BuOOH treatment no protection was afforded, and the cells were more prone to damage by t-BuOOH at 150 μM ($p < 0.05$) and 250 μM ($p < 0.01$).

In a second experiment, Figure 3.19b, the above results were confirmed. In EAhy926 cells which had been pre-cultured in 40 nM sodium selenite, and which also received selenite (40 nM) simultaneously with the t-BuOOH, total protection from toxicity from all concentrations of t-BuOOH tested (25 to 250 μM) was observed ($p < 0.05$), apart from 100 μM. In EAhy926 cells to which sodium selenite (40 nM) was added at the same time as t-BuOOH treatment, but not as a pre-incubation, no protection was afforded, and the cells were seen to be more sensitive to damage by 75 μM t-BuOOH than were the control cells that received no selenite supplementation at any time, although the increase in damage was not significant.

A third experiment showed EAhy926 cells pre-cultured in 40nM sodium selenite, and which also received selenite (40 nM) simultaneously with the t-BuOOH to be protected from t-BuOOH toxicity produced by concentrations of 25 μM to 100 μM (figure 3.19c). There was a small but significant protective effect at 150 μM t-BuOOH, but all protection was lost by 200 μM t-BuOOH. In EAhy926 cells to which sodium selenite (40 nM) was added at the same time as t-BuOOH treatment, again no protection was afforded, and the cells were seen to have increased sensitivity to damage by t-BuOOH. The increased susceptibility was not significant however.

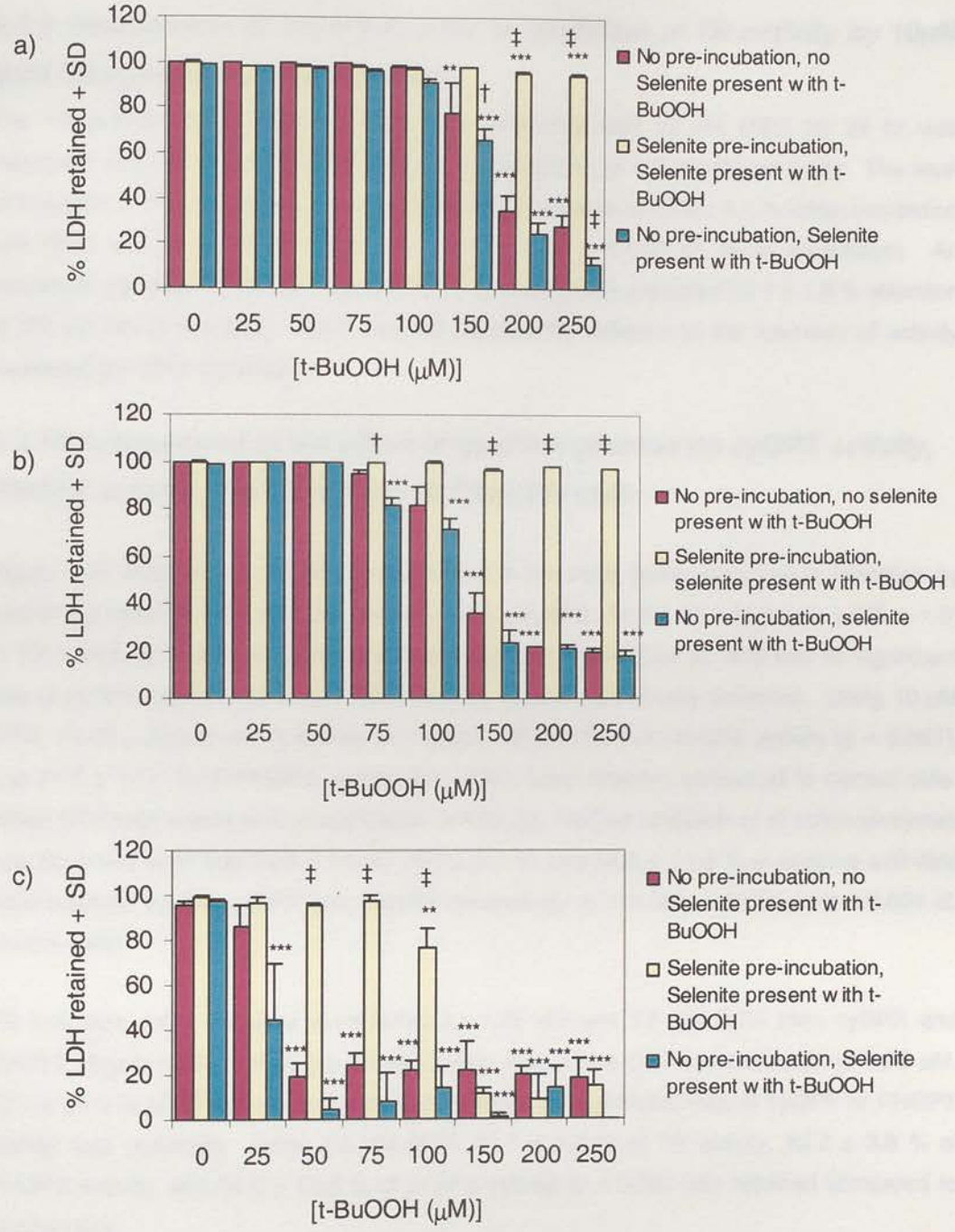


Figure 3.19 The effects of sodium selenite on the sensitivity of EAhy926 cells to tert-butylhydroperoxide (t-BuOOH) cytotoxicity added either prior to t-BuOOH treatment or at the same time as t-BuOOH treatment. EAhy926 cells were pre-incubated with either Se-deficient medium supplemented with sodium selenite (40 nM) or Se-deficient medium with no addition. After 48 hr incubation the medium was removed and replaced with medium containing 40 nM sodium selenite and t-BuOOH (0 to 250 μM) or t-BuOOH unsupplemented with selenite for 20 hr. Control cells received no t-BuOOH or Se supplementation. Results shown are the mean of triplicate wells + SD. $p < 0.01$**, $p < 0.001$*** cf. control cells for each respective treatment group. $p < 0.05$†, $p < 0.01$‡ cf cells which did not receive selenite pre-incubation or in conjunction with t-BuOOH, receiving the same t-BuOOH concentration. Graphs (a) – (c) are separate experiments.

3.3.9 Assessment of the timecourse of inhibition of TR activity by 10μM gold thioglucose in EAhy926 cells

The TR activity retained by EAhy926 cells incubated with 10 μM GTG for 24 hr was measured at 63.2 ± 13.6 % (mean ± SD, n = 3) retention ($p < 0.01$) (figure 3.20). The level of retention of TR activity was seen to significantly decrease to 36.6 ± 4.0 % when incubation with GTG was for 48 hr ($p < 0.001$ cf. control cells; $p < 0.05$ cf. 24 hr incubation). An incubation period of 72 hr with 10 μM GTG in EAhy926 cells produced 29.1 ± 1.9 % retention of TR activity ($p < 0.001$), which was not significantly different to the retention of activity measured for 48 hr incubation.

3.3.10 Assessment of the effect of gold thioglucose on cyGPX activity, PHGPX activity and TR activity of EAhy926 cells

Figure 3.21 illustrates the differing sensitivities of the three selenoenzymes to inhibition by gold thioglucose (GTG). At a concentration of 1 μM GTG, 74.8 ± 11.1 % (mean ± SD, n = 6) of TR activity ($p < 0.01$ cf. control cells) was retained (figure 3.21 a), whereas no significant loss of cyGPX (figure 3.21 c) or PHGPX activity (figure 3.21 b) was observed. Using 10 μM GTG, 14.02 ± 20.9 % of TR activity ($p < 0.001$), 40.2 ± 5.6 % of cyGPX activity ($p < 0.001$), and 77.5 ± 17.7 % of PHGPX activity ($p < 0.01$) were retained compared to control cells. When GTG was added at a concentration of 100 μM, marked inhibition of all selenoenzymes was observed such that 0.56 ± 1.4 %, 15.1 ± 2.7 %, and 54.4 ± 11.3 % of enzyme activities were retained for TR, cyGPX and PHGPX respectively ($p < 0.001$, < 0.001, and < 0.001 cf. control cells).

TR was also more sensitive to inhibition by 1.75 μM and 2.5 μM GTG than cyGPX and PHGPX (figure 3.22). When the pre-incubation was with a GTG concentration of 1.75 μM, 75.1 ± 23.0 % of TR activity was retained, whereas no significant loss of cyGPX or PHGPX activity was observed. Using 2.5 μM GTG, 71.7 ± 3.2 % of TR activity, 82.2 ± 3.9 % of PHGPX activity, and 66.0 ± 10.6 % of cyGPX activity ($p < 0.05$) was retained compared to control cells.

Supplementation of cells with GTG at concentrations of 1 μM or 10 μM did not alter the TR concentration of the cells compared to control cells as assessed by TR RIA (data not shown). There are no methods currently available to us to assess cyGPX concentration.

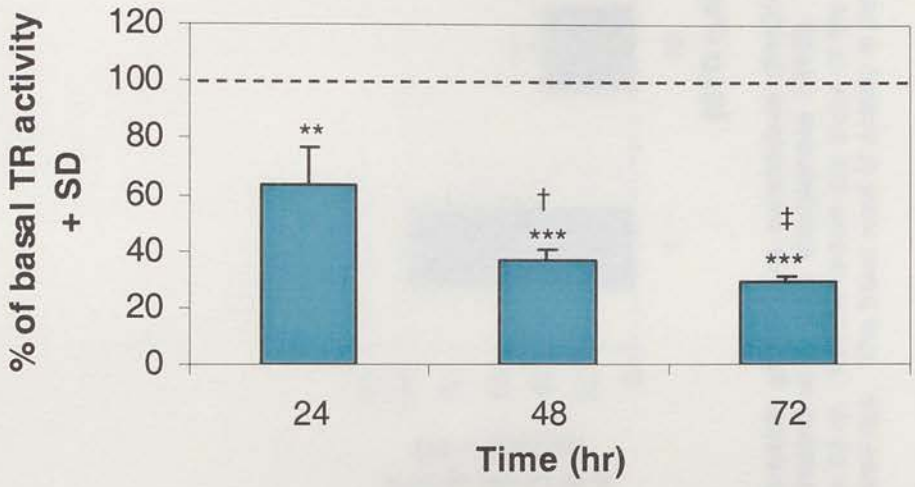


Figure 3.20 Timecourse of inhibition of thioredoxin reductase (TR) activity by gold thioglucose (GTG) in EAhy926 cells. Cells were incubated with Se-deficient medium supplemented with GTG (10 μM) for 24, 48, and 72 hr respectively. Results shown are those of the mean of 3 flasks + SD. The basal level of activity in control cells untreated with GTG is indicated by the dashed line. $p < 0.01$ **; $p < 0.001$ *** cf. control cells. $p < 0.05$†, $p < 0.01$‡ cf. TR activity of GTG-treated cells at 24 hr.

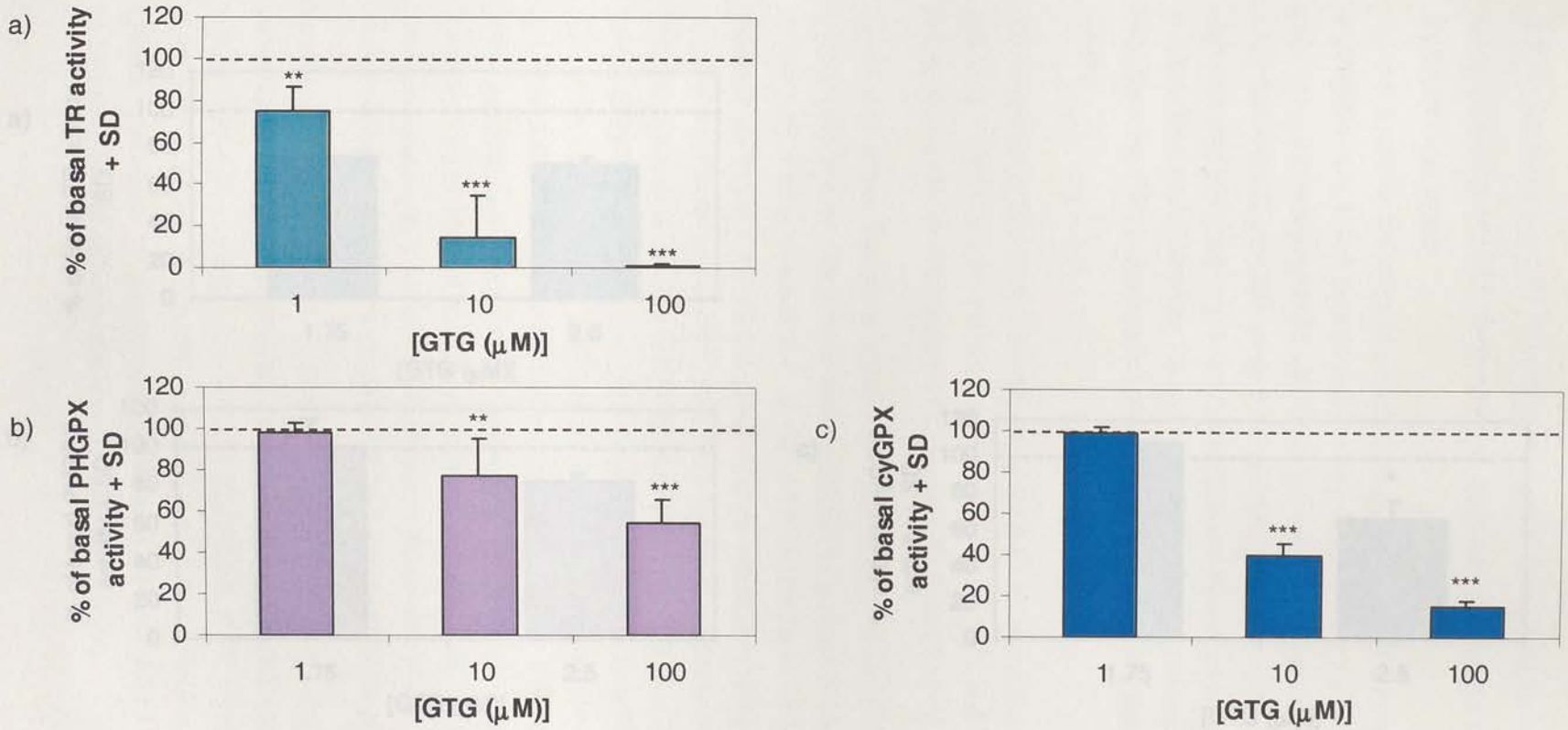


Figure 3.21 Thioredoxin reductase (TR) activity (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in EAhy926 cells treated with gold thioglucose (GTG). EAhy926 cells were incubated with Se-deficient medium supplemented with GTG (0, 1, 10, 100 μM) for 48 hr. Results shown are those of the mean of 3 flasks per experiment, of 2 separate experiments meaned, assayed in the same run on the same day. The basal level of activity is indicated by the dashed line. $p < 0.01$ **; $p < 0.001$ *** cf. control cells.

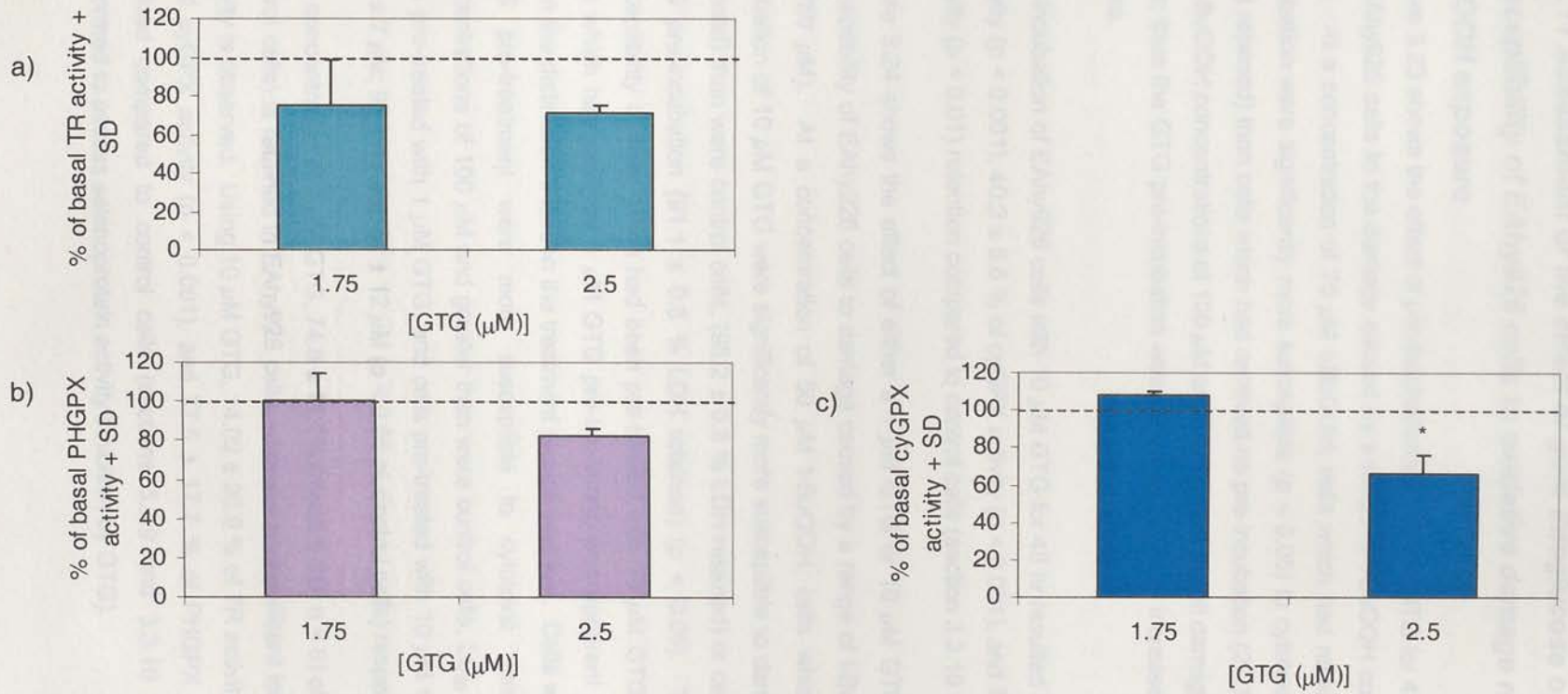


Figure 3.22 Thioredoxin reductase (TR) activity (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in EAhy926 cells treated with gold thioglucose (GTG). EAhy926 cells were incubated with Se-deficient medium supplemented with GTG (0, 1.75, 2.5 μM) for 48 hr. Results shown are those of the mean of triplicate flasks + SD. The basal level of activity in control cells is indicated by the dashed line. $p < 0.01$ **; $p < 0.001$ *** cf. control cells.

3.3.11 Assessment of the effect of gold thioglucose on the susceptibility of EAhy926 cells to oxidative damage resulting from t-BuOOH exposure

Figure 3.23 shows the effect of pre-incubation with 10 μM GTG for 48 hr on the susceptibility of EAhy926 cells to the damage caused by a range of t-BuOOH concentrations (0, 75, 100 μM). At a concentration of 75 μM t-BuOOH, cells which had received a 10 μM GTG pre-incubation were significantly more susceptible ($p < 0.05$) to cytotoxic damage (4.4 ± 1.6 % LDH retained) than cells which had received no pre-incubation (23.3 ± 3.7 % LDH retained). At t-BuOOH concentrations of 100 μM and above, >95 % cell damage occurred in the control cells; thus the GTG pre-incubation was unable to show an increased susceptibility at these doses.

Pre-incubation of EAhy926 cells with 10 μM GTG for 48 hr resulted in 14.02 ± 20.9 % of TR activity ($p < 0.001$), 40.2 ± 5.6 % of cyGPX activity ($p < 0.001$), and 77.5 ± 17.7 % of PHGPX activity ($p < 0.01$) retention compared to control cells (section 3.3.10 above).

Figure 3.24 shows the effect of either a 1 μM GTG or 10 μM GTG pre-incubation on the susceptibility of EAhy926 cells to damage caused by a range of t-BuOOH concentrations (0 to 250 μM). At a concentration of 50 μM t-BuOOH, cells which had received a pre-incubation of 10 μM GTG were significantly more susceptible to damage (68.3 ± 5.5 % LDH retained) than were control cells, (95.2 ± 0.8 % LDH retained) or cells which received 1 μM GTG pre-incubation (91.1 ± 0.8 % LDH retained) ($p < 0.05$). This trend of increased susceptibility of cells which had been pre-treated with 10 μM GTG over control cells, and cells which had received 1 μM GTG pre-treatment, was apparent until 200 μM t-BuOOH, when the distinction between the treatment groups was lost. Cells which had received 1 μM GTG pre-treatment were more susceptible to cytotoxic damage by t-BuOOH at concentrations of 100 μM and greater than were control cells. LC_{50} values for control cells, cells pre-treated with 1 μM GTG, and cells pre-treated with 10 μM GTG were calculated as 112 ± 7 μM; 93 ± 13 μM; 62 ± 12 μM ($p < 0.05$ cf. control cells) respectively.

At a concentration of 1 μM GTG, 74.8 ± 11.1% (mean ± SD, n = 6) of TR activity ($p < 0.01$ cf. control cells) is retained in EAhy926 cells, whereas no significant loss of cyGPX or PHGPX activity is observed. Using 10 μM GTG, 14.02 ± 20.9 % of TR activity ($p < 0.001$), 40.2 ± 5.6 % of cyGPX activity ($p < 0.001$), and 77.5 ± 17.7 % of PHGPX activity ($p < 0.01$) was retained compared to control cells (sections 3.3.9 and 3.3.10 detail the experiments performed to assess selenoprotein activity inhibition by GTG).

Concentrations of 1.75 μM and 2.5 μM GTG were investigated to determine whether they would offer further selectivity of inhibition of TR activity without affecting the activity of cyGPX and PHGPX. Figure 3.25 shows the effect of either a 1.75 μM or 2.5 μM GTG pre-incubation on the susceptibility of EAhy926 cells to the damage caused by a range of t-BuOOH concentrations (0 to 300 μM). Cells pre-incubated with either 1.75 μM or 2.5 μM GTG showed similar susceptibility to cytotoxic damage from all the concentrations of t-BuOOH tested. Significant cytotoxicity was first sustained at a concentration of 75 μM t-BuOOH ($p < 0.001$) when cells received a pre-incubation of 1.75 μM or 2.5 μM GTG, whereas control cells first showed cytotoxicity at 150 μM t-BuOOH. There were no significant differences in the susceptibilities of the cells that received either 1.75 μM or 2.5 μM GTG to cytotoxicity at any concentration of t-BuOOH.

Using these GTG concentrations, 1.75 μM GTG resulted in 75.1 ± 23.0 % of TR activity retention, whereas no significant loss of cyGPX or PHGPX activity was observed. Using 2.5 μM GTG, 71.7 ± 3.2 % of TR activity, 82.2 ± 3.9 % of PHGPX activity, and 66.0 ± 10.6 % of cyGPX activity ($p < 0.05$) was retained compared to control cells (figure 3.22).

Using a range of GTG concentrations for pre-incubation of the EAhy926 cells (1, 2.5, 5, 7.5, and 10 μM GTG) demonstrated a trend for increasing damage mediated by t-BuOOH with increasing GTG concentration (figure 3.26). At a t-BuOOH concentration of 150 μM, it was demonstrated that the higher the GTG concentration used for the pre-incubation of the EAhy926 cells, the more damage was sustained from the t-BuOOH. However, the differences between the cells receiving the differing GTG concentrations were not significant due to large variations between the amount of LDH released from individual wells. The trend for increasing susceptibility was partially demonstrated at a concentration of 200 μM t-BuOOH, although the differential susceptibility between the higher GTG concentrations had been lost at this concentration. Again, the large variations in the data meant that differences between the treatment groups were not significant. A GTG concentration of 1 μM used for pre-treatment did not significantly increase the susceptibility of the cells to damage by t-BuOOH at any of the concentrations tested, although it had been demonstrated to do so in a previous experiment (figure 3.24).

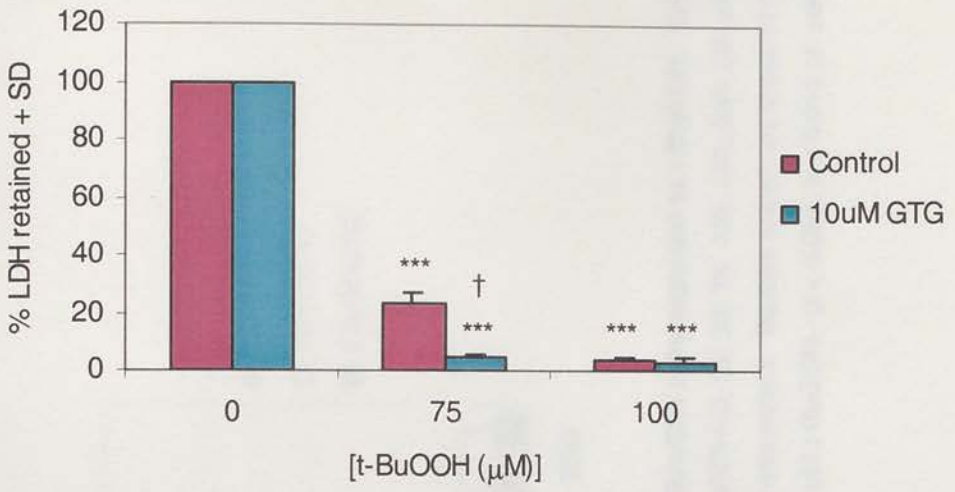


Figure 3.23 The effect of gold thioglucose pre-incubation on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with 10 μM gold thioglucose (GTG) for 48 hr prior to exposure to t-BuOOH (0, 75, 100 μM) for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.001$*** cf. control cells receiving 0 μM t-BuOOH. $p < 0.05$† cf. control cells receiving the same concentration of t-BuOOH.

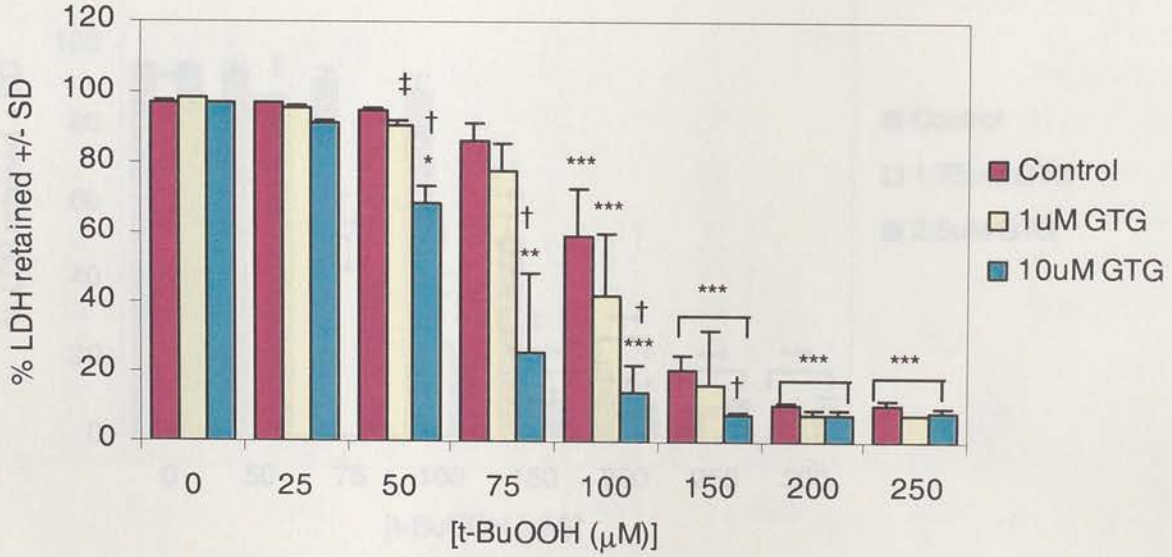


Figure 3.24 The effect of gold thioglucose pre-incubation on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with 1 μM or 10 μM gold thioglucose (GTG) for 48 hr prior to exposure to t-BuOOH (0, 25, 50, 75, 100, 150, 200, 250 μM) for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. respective control cells receiving 0 μM t-BuOOH. $p < 0.05$†; $p < 0.01$‡ cf. control cells at the same concentration of t-BuOOH.

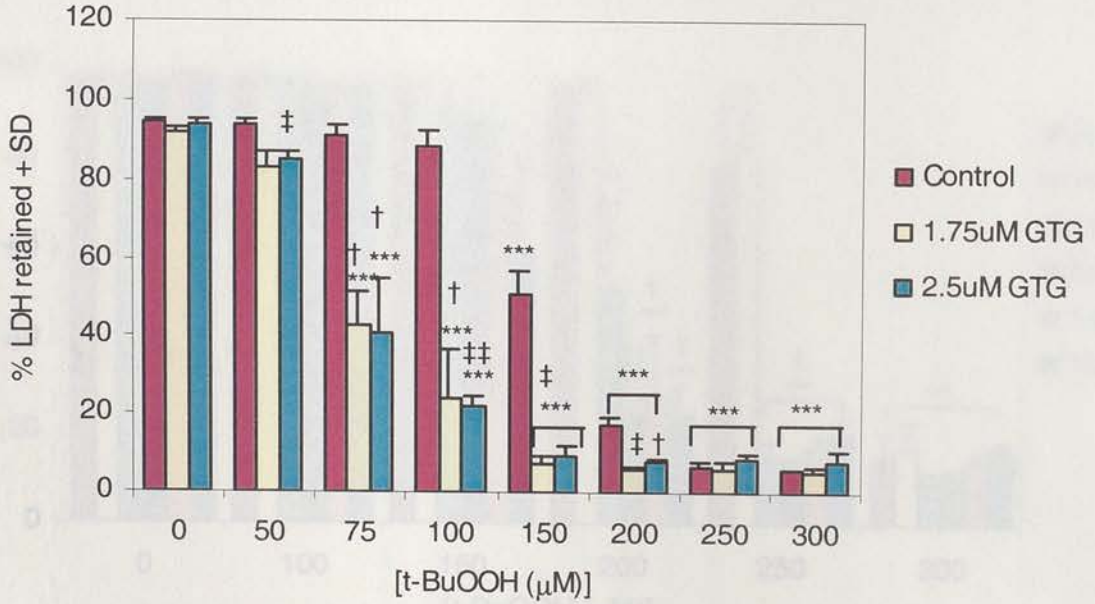


Figure 3.25 The effect of gold thioglucose pre-incubation on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with 1.72 μM or 2.5 μM gold thioglucose (GTG) for 48 hr prior to exposure to t-BuOOH (0, 50, 75, 100, 150, 200, 250, 300 μM) for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.001$*** cf. respective control cells of each treatment group. $p < 0.05$ †, $p < 0.01$‡, $p < 0.001$‡‡ cf. control cells treated with the same t-BuOOH concentration.

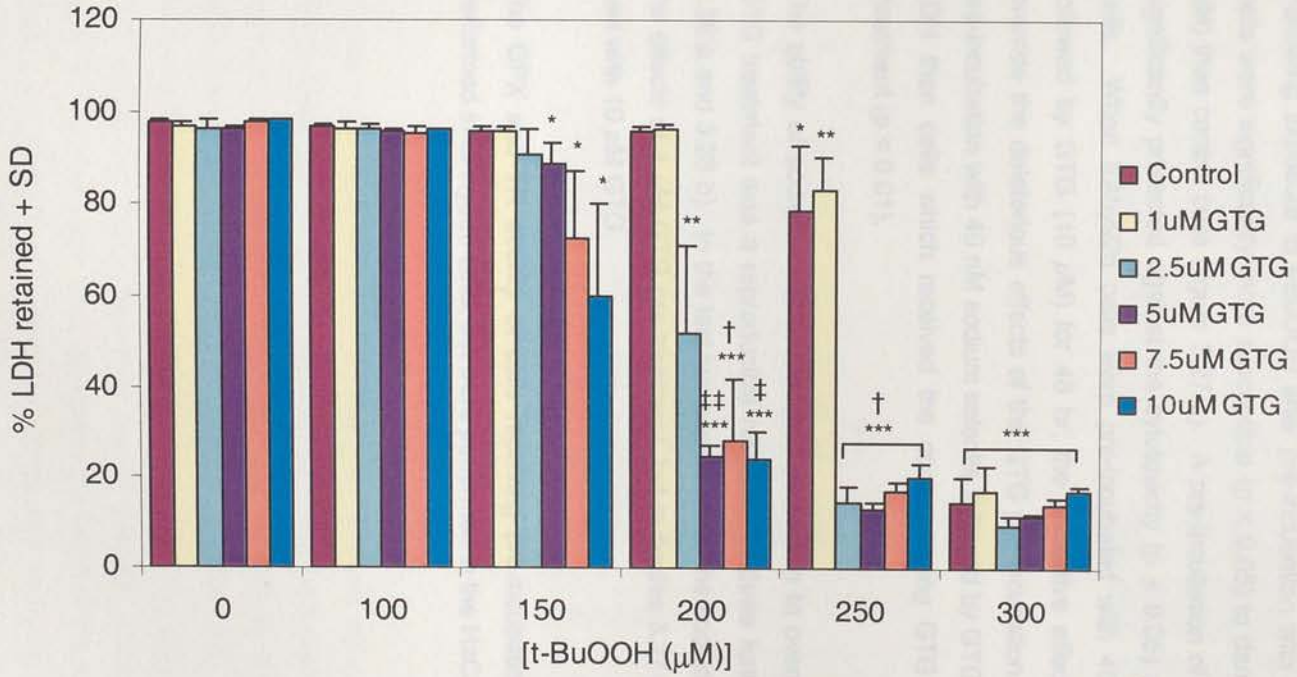


Figure 3.26 The effect of gold thioglucose pre-incubation on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with gold thioglucose (GTG) (1, 2.5, 5, 7.5, 10 μM) for 48 hr prior to exposure to t-BuOOH (0, 100, 150, 200, 250, 300 μM) for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p< 0.01$**, $p < 0.001$*** cf. respective control cells receiving 0 μM t-BuOOH. $p < 0.05$†, $p < 0.01$‡, $p < 0.001$‡‡ cf. control cells treated with the same t-BuOOH concentration.

3.3.12 Assessment of the effect of consecutive sodium selenite and gold thioglucose treatment on susceptibility of EAhy926 cells to oxidative damage resulting from t-BuOOH exposure

Following exposure to t-BuOOH after pre-incubation with 10 μM GTG for 48 hr, EAhy926 cells were significantly more susceptible ($p < 0.05$) to damage by t-BuOOH (40 μM to 100 μM) than control cells (figure 3.27 a). A pre-incubation of 40 nM sodium selenite for 48 hr significantly protected against the cytotoxicity ($p < 0.05$) sustained by Se-deficient control cells. When EAhy926 cells were pre-incubated with 40 nM sodium selenite for 48 hr followed by GTG (10 μM) for 48 hr, the protective effects of the selenite were seen to override the deleterious effects of the GTG pre-incubation. Thus, the cells which received pre-incubation with 40 nM sodium selenite followed by GTG retained a significantly higher % LDH than cells which received the corresponding GTG concentration alone as a pre-treatment ($p < 0.01$).

The ability of sodium selenite-mediated protection to override the deleterious effect of the GTG treatment was a reproducible response in three further experiments (figures 3.27 b, 3.28 a and 3.28 b). In the last two experiments, the sodium selenite was used to overcome the effects of 1 μM GTG pre-treatment, but in figures 3.27 a and 3.27 b the GTG treatment was with 10 μM GTG.

The GPX and TR activity of cells receiving pre-incubations as detailed above were not performed in EAhy926 cells, but were performed in the HaCaT cell line (see section 4.3.10).

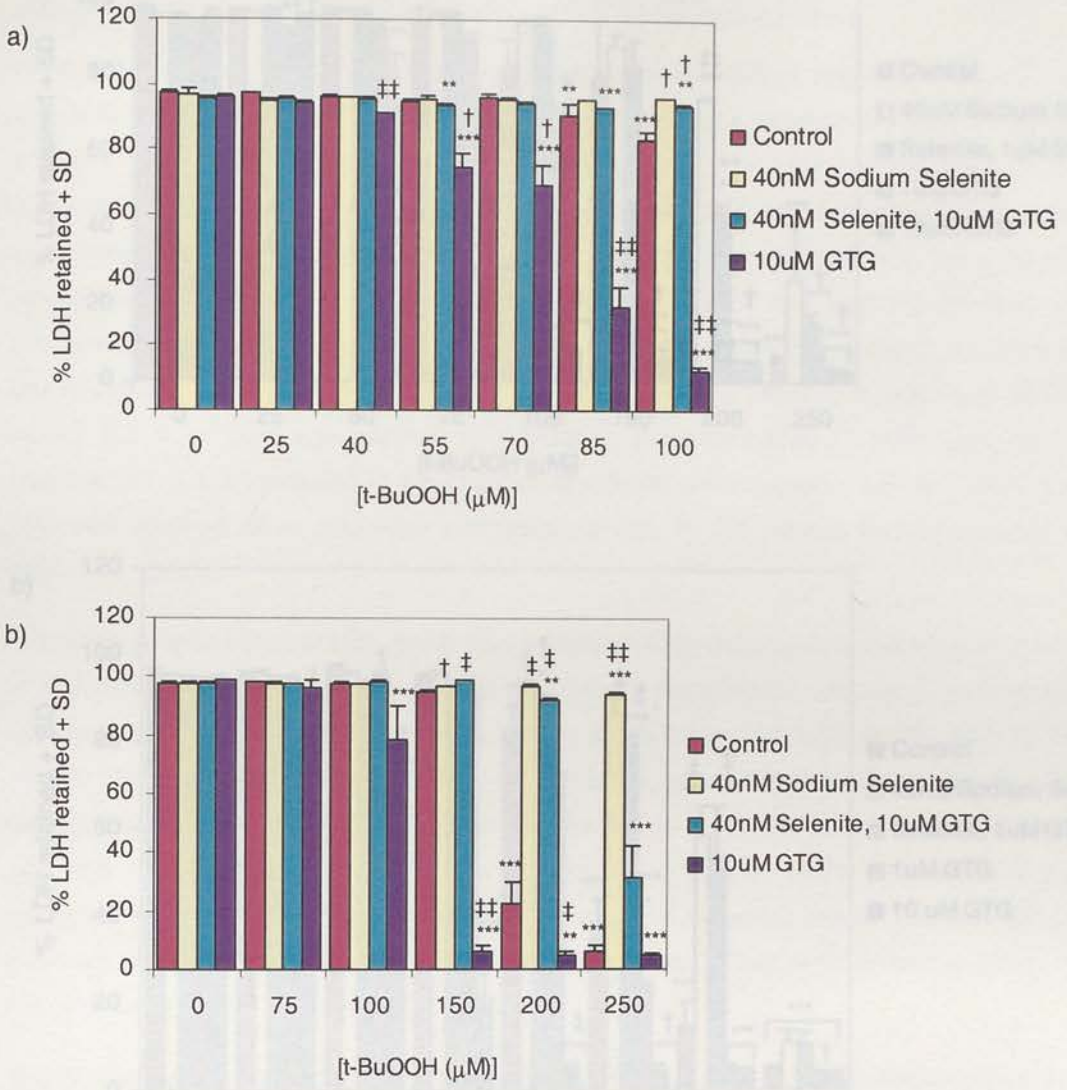


Figure 3.27 The effect of consecutive sodium selenite and gold thioglucose pre-incubations on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with sodium selenite (40 nM) followed by gold thioglucose (GTG) (10 μM), or either one of these pre-incubations, each for 48 hr. Cells were washed between and following pre-incubations. Exposure to t-BuOOH (0 - 100 μM, or 0 – 250 μM) was for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a selenite or GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.01^{**}$, $p < 0.001^{***}$ cf. respective control cells receiving 0 μM t-BuOOH. $p < 0.05$†, $p < 0.01$‡, $p < 0.001$‡‡ cf. control cells receiving the same t-BuOOH concentration. Graphs (a) and (b) are each a separate experiment.

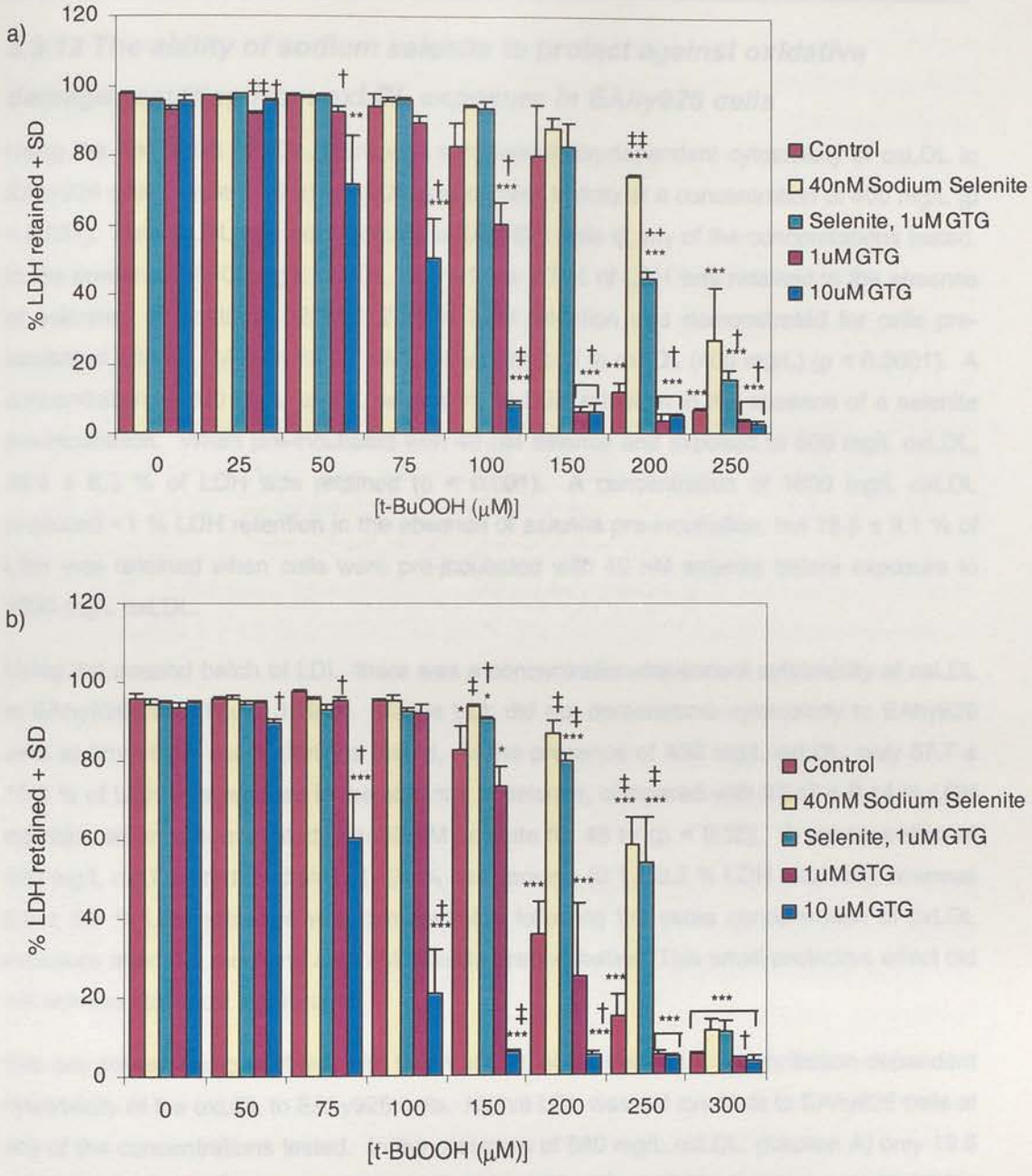


Figure 3.28 The effect of consecutive sodium selenite and gold thioglucose pre-incubations on the % LDH retained by EAhy926 cells exposed to t-BuOOH. EAhy926 cells were pre-treated with sodium selenite (40 nM) followed by gold thioglucose (GTG) (1 μM), or either one of these pre-incubations, or 10 μM GTG, each for 48 hr. Cells were washed between and following pre-incubations. Exposure to t-BuOOH (0 - 250 μM or 300 μM) was for 20 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a selenite or GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. respective control cells receiving 0 μM t-BuOOH. $p < 0.05$ †, $p < 0.01$ ‡, $p < 0.001$‡‡ cf. control cells receiving the same t-BuOOH concentration. Graphs (a) and (b) are each a separate experiment.

3.3.13 The ability of sodium selenite to protect against oxidative damage resulting from oxLDL exposure in EAhy926 cells

Using the first batch of LDL, there was a concentration-dependent cytotoxicity of oxLDL to EAhy926 cells (figure 3.29 a). OxLDL first showed toxicity at a concentration of 400 mg/L ($p < 0.001$). Native LDL was not cytotoxic to EAhy926 cells at any of the concentrations tested. In the presence of 400 mg/L oxLDL, only 31.1 ± 3.7 % of LDH was retained in the absence of selenite. In contrast, 92.27 ± 2.06 % LDH retention was demonstrated for cells pre-incubated with 40 nM selenite for 48 hr when exposed to oxLDL (400 mg/L) ($p < 0.0001$). A concentration of 800 mg/L oxLDL caused <1 % LDH retention in the absence of a selenite pre-incubation. When pre-incubated with 40 nM selenite and exposed to 800 mg/L oxLDL, 38.4 ± 8.3 % of LDH was retained ($p < 0.001$). A concentration of 1600 mg/L oxLDL produced <1 % LDH retention in the absence of selenite pre-incubation, but 18.8 ± 9.1 % of LDH was retained when cells were pre-incubated with 40 nM selenite before exposure to 1600 mg/L oxLDL.

Using the second batch of LDL, there was a concentration-dependent cytotoxicity of oxLDL to EAhy926 cells (figure 3.29 b). Native LDL did not demonstrate cytotoxicity to EAhy926 cells at any of the concentrations tested. In the presence of 480 mg/L oxLDL, only 57.7 ± 10.3 % of LDH was retained in the absence of selenite, compared with 97.47 ± 0.14 % LDH retention when pre-incubated with 40 nM selenite for 48 hr ($p < 0.05$). A concentration of 960 mg/L oxLDL produced almost 100 % cell damage (0.1 ± 0.2 % LDH retained), whereas 6.9 ± 5.6 % LDH retention was demonstrated following the same concentration of oxLDL exposure after first receiving a 40 nM selenite pre-incubation. This small protective effect did not achieve statistical significance.

The two paired fractions of the third batch of LDL also exhibited a concentration-dependent cytotoxicity of the oxLDL to EAhy926 cells. Native LDL was not cytotoxic to EAhy926 cells at any of the concentrations tested. In the presence of 560 mg/L oxLDL, (fraction A) only 19.8 ± 1.8 % of LDH was retained in the absence of selenite (figure 3.30 a), compared with 92.7 ± 0.4 % LDH retention by cells pre-incubated with 40 nM selenite for 48 hr and exposed to oxLDL (560 mg/L) ($p < 0.001$). A concentration of 1120 mg/L oxLDL produced more cytotoxicity than 560 mg/L, demonstrating only 2.5 ± 0.4 % LDH retention in the absence of a selenite pre-incubation. When pre-incubated with 40 nM selenite and exposed to 1120 mg/L oxLDL, 2.5 ± 0.8 % of LDH was retained, indicating that the level of cell damage produced by a concentration of 1120 mg/L oxLDL was too extensive to protect against.

In the presence of 560 mg/L oxLDL, (fraction B) only 17.5 ± 2.2 % of LDH was retained in the absence of selenite (figure 3.30 b). Cells pre-incubated with 40 nM selenite for 48 hr retained 93.1 ± 1.4 % LDH in comparison when exposed to oxLDL (560 mg/L) ($p < 0.001$). A

concentration of 1120 mg/L oxLDL produced further cytotoxicity than 560 mg/L, resulting in 7.2 ± 0.3 % LDH retention in the absence of a selenite pre-incubation. When pre-incubated with 40 nM selenite and exposed to 1120 mg/L oxLDL, 7.3 ± 0.9 % of LDH was retained, indicating that the level of cell damage produced by 1120 mg/L oxLDL was too extensive to protect against.

3.3.14 The effect of sodium selenite and/or gold thioglucose pre-incubation on the susceptibility of EAhy926 cells to oxidative damage resulting from oxLDL exposure

OxLDL demonstrated a concentration dependent cytotoxicity to EAhy926 cells, producing significant cytotoxicity at 220, 440, and 880 mg/L ($p < 0.001$) (figure 3.31 a). In the presence of 220 mg/L oxLDL, Se-deficient control cells retained 70.4 ± 2.5 % of their LDH activity, compared to 59.8 ± 5.1 % LDH activity retention by cells pre-incubated with 1 μM GTG for 48 hr ($p < 0.05$). At the same dose of oxLDL, cells pre-incubated with 40 nM sodium selenite for 48 hr retained 99.1 ± 1.6 % of their LDH, which was highly significant protection compared to Se-deficient cells ($p < 0.0001$). Cells which were pre-treated with 40 nM sodium selenite for 48 hr followed by 1 μM GTG for 48 hr prior to exposure to 220 mg/L oxLDL demonstrated 98.5 ± 0.9 % LDH retention, which was not significantly different to the % LDH retention by cells which received sodium selenite pre-incubation alone. This protection was highly significant also ($p < 0.0001$).

At a concentration of 440 mg/L oxLDL, EAhy926 cells pre-incubated with 1 μM GTG showed increased susceptibility to damage, retaining 6.2 ± 0.5 % LDH in comparison to controls which retained 10.0 ± 0.2 % LDH ($p < 0.0001$). Cells which received a pre-incubation with 40 nM sodium selenite retained 22.8 ± 4.8 % LDH, which was significant protection in comparison to Se-deficient control cells ($p < 0.05$). The % LDH retention by cells pre-incubated with 40 nM sodium selenite alone was not significantly different to the level retained by cells which received 40 nM sodium selenite for 48 hr followed by 1 μM GTG for 48 hr as pre-incubations. Thus, the sodium selenite pre-incubation was able to overcome the deleterious effect of the GTG pre-incubation.

Cytotoxicity of oxLDL to the EAhy926 cells was first evident at a concentration of 220 mg/L, producing 70.4 ± 2.5 % LDH retention in control cells. At this oxLDL concentration, total glutathione was depleted in the cells so as to be beyond the lower detection limit of our assay (figure 3.31 b). Depletion of total glutathione was not evident at any of the concentrations of oxLDL studied below 220 mg/L. There were no significant differences in the total glutathione level of cells in any of the treatment groups between different oxLDL concentrations.

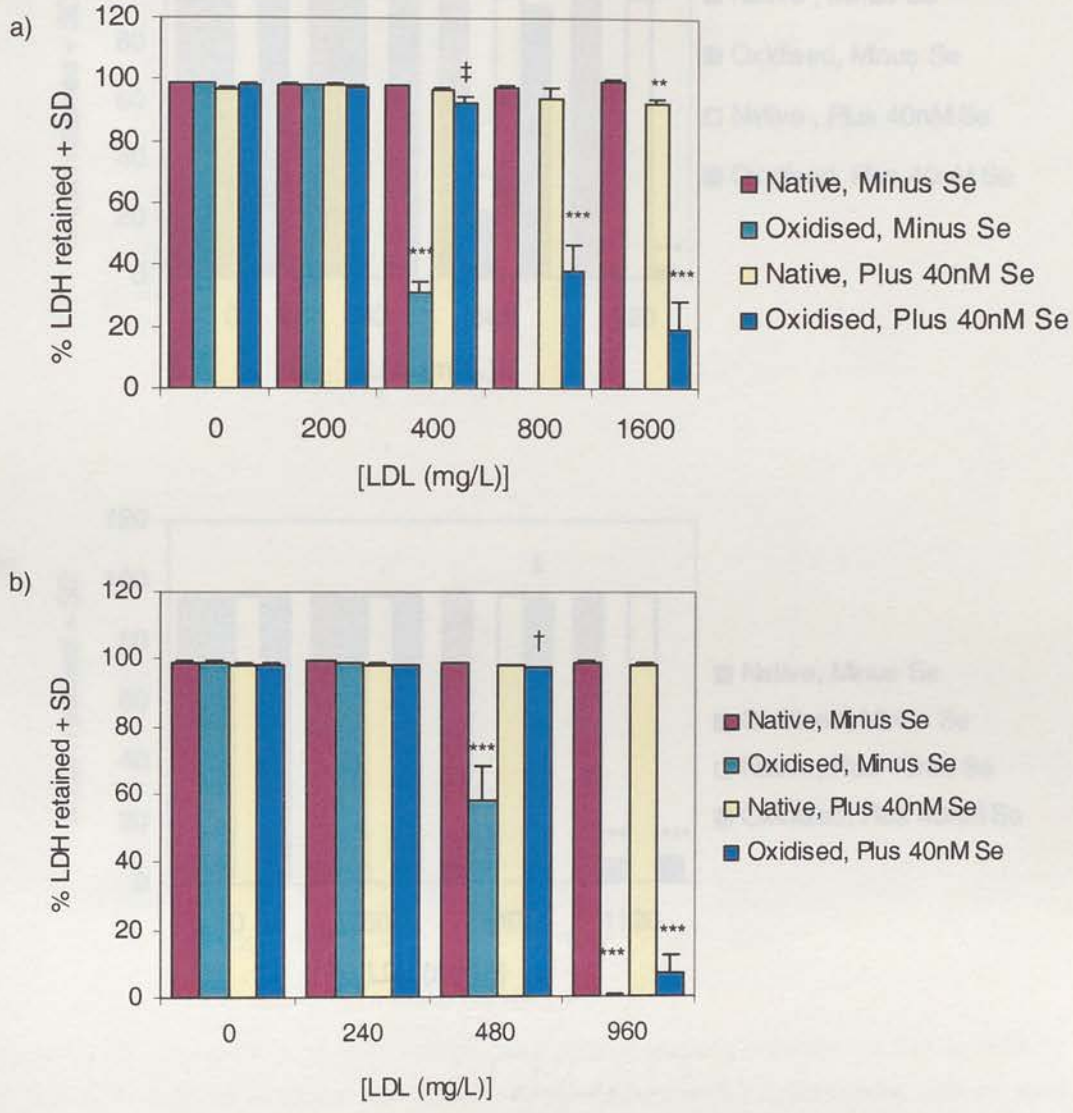


Figure 3.29 The effects of sodium selenite on the sensitivity of EAhy926 cells to oxidised and native low-density lipoprotein (LDL) cytotoxicity. EAhy926 cells were pre-incubated with either Se-deficient medium supplemented with sodium selenite (40 nM) or Se-deficient medium with no addition. After 48 hr incubation the medium was removed, the cells washed, and the cells received medium containing native LDL or oxidised LDL (oxLDL) (0, 200, 400, 800, 1600 μM) (experiment 'a') or (0, 240, 480, 960 μM) (experiment 'b') for 24 hr. Control cells received no Se supplementation. Results shown are the mean of triplicate wells + SD. $p < 0.01$**, $p < 0.001$*** cf. respective control cells receiving 0 mg/L LDL. $p < 0.05$†, $p < 0.0001$‡ cf. Se-deficient cells treated with the same concentration of oxLDL. Graphs (a) and (b) are each a separate experiment using a different preparation of LDL.

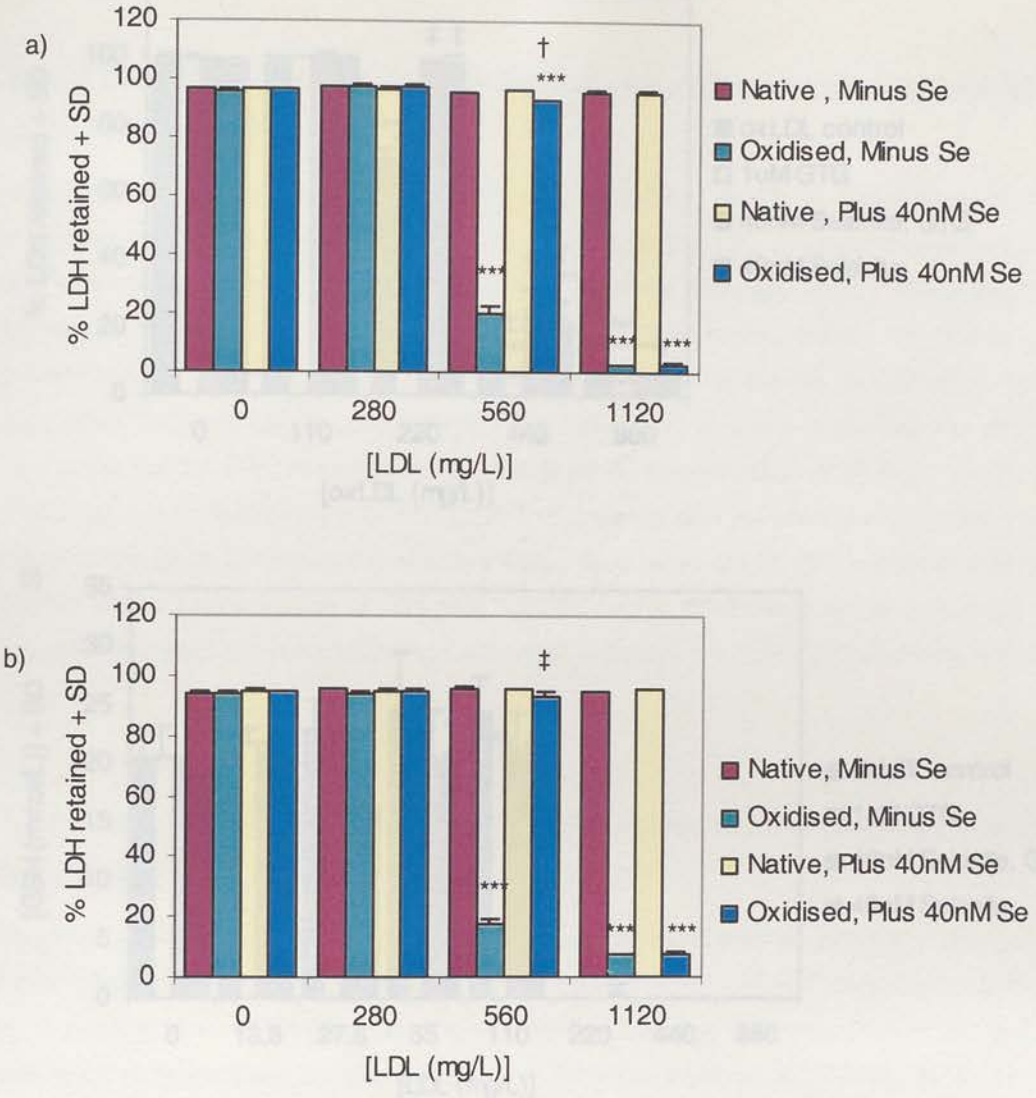


Figure 3.30 The effects of sodium selenite on the sensitivity of EAhy926 cells to oxidised and native low-density lipoprotein (LDL) cytotoxicity. EAhy926 cells were pre-incubated with either Se-deficient medium supplemented with sodium selenite (40 nM) or Se-deficient medium with no addition. After 48 hr incubation the medium was removed, the cells washed, and the cells received medium containing native LDL or oxidised LDL (oxLDL) (0, 280, 560, 1120 μM) for 24 hr. Control cells received no Se supplementation. Results shown are the mean of triplicate wells + SD. $p < 0.001$*** cf. respective control cells receiving 0 mg/L LDL. $p < 0.001$ †, $p < 0.0001$‡ cf. Se-deficient cells treated with the same concentration of oxLDL. Graphs (a) and (b) are each a different experiment with a paired fraction of the same LDL preparation.

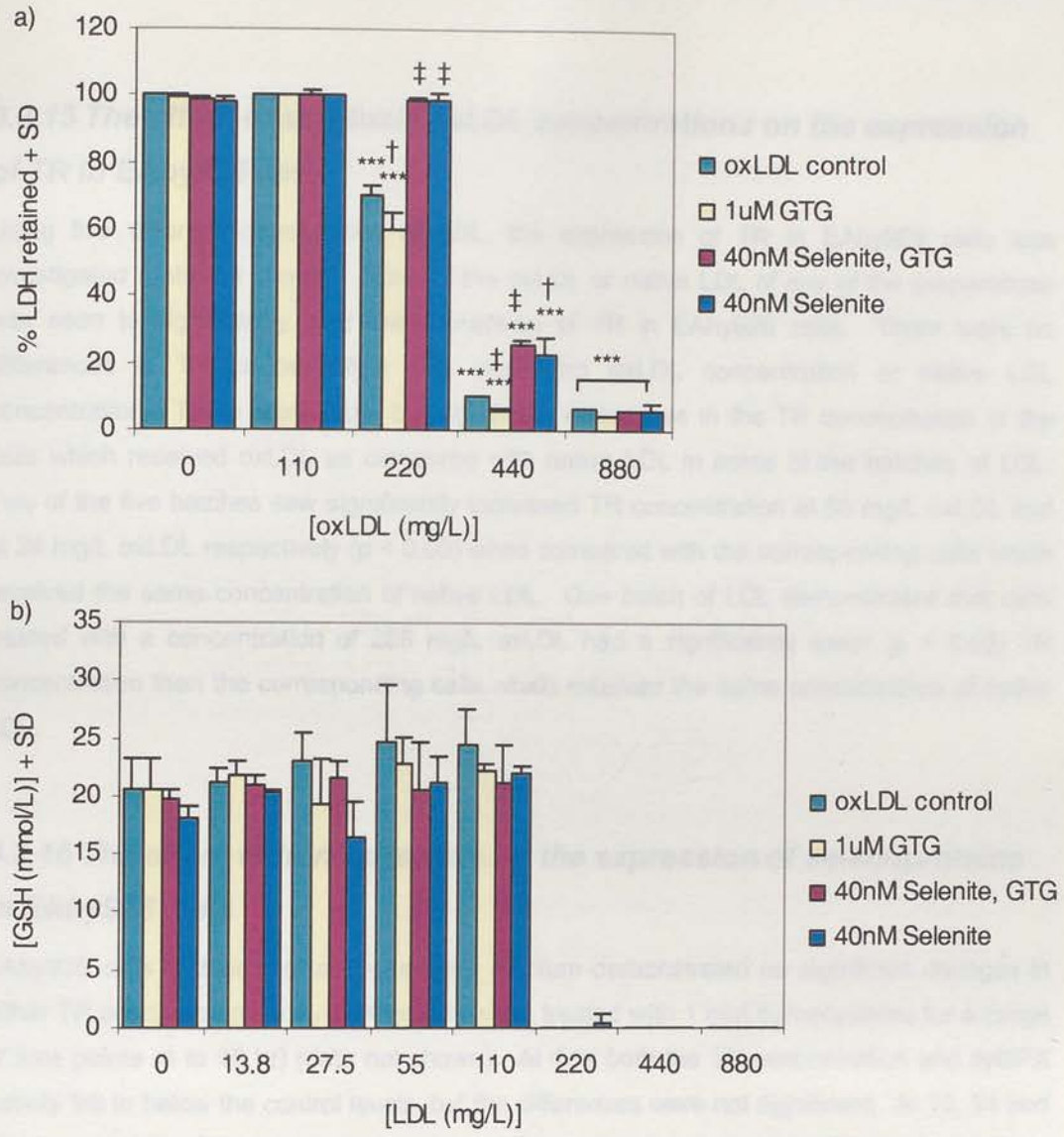


Figure 3.31 The effects of gold thioglucose and/or sodium selenite on the sensitivity of EAhy926 cells to oxidised low-density lipoprotein (oxLDL) cytotoxicity, and on total glutathione status. EAhy926 cells were pre-incubated for 48 hr with Se-deficient medium supplemented with sodium selenite (40 nM) or 1μM gold thioglucose (GTG), or selenite followed by GTG. After pre-incubation the medium was removed, the cells washed, and the cells received medium containing oxidised LDL (oxLDL) (0, 13.8, 27.5, 55, 110, 220, 440, 880 μM) for 24 hr. Control cells received no Se or gold thioglucose supplementation. Cytotoxicity was assessed by % LDH activity retained. For glutathione assay, cells were harvested into 3.3 % sulphosalicylic acid, and lysed by 2 freeze-thaw cycles at -80°C. Results shown are the mean of triplicate wells + SD. $p < 0.001$*** cf. control cells receiving the respective pre-incubation, but no oxLDL. $p < 0.05$†, $p < 0.0001$‡ cf. control cells receiving the same oxLDL concentration. Graphs (a) and (b) are data from one experiment, graph (a) detailing cytotoxicity, and graph (b) detailing total glutathione status.

3.3.15 The effect of sub-toxic oxLDL concentrations on the expression of TR in EAhy926 cells

Using five different preparations of LDL, the expression of TR in EAhy926 cells was investigated (data not shown). None of the oxLDL or native LDL of any of the preparations was seen to significantly alter the expression of TR in EAhy926 cells. There were no differences in TR concentration with increasing oxLDL concentration or native LDL concentration. There were slight but significant differences in the TR concentration of the cells which received oxLDL as compared with native LDL in some of the batches of LDL. Two of the five batches saw significantly increased TR concentration at 50 mg/L oxLDL and at 24 mg/L oxLDL respectively ($p < 0.05$) when compared with the corresponding cells which received the same concentration of native LDL. One batch of LDL demonstrated that cells treated with a concentration of 200 mg/L oxLDL had a significantly lower ($p < 0.05$) TR concentration than the corresponding cells which received the same concentration of native LDL.

3.3.16 The effect of homocysteine on the expression of selenoproteins in EAhy926 cells

EAhy926 cells in their normal Se-deficient medium demonstrated no significant changes in either TR concentration or cyGPX activity when treated with 1 mM homocysteine for a range of time points (4 to 48 hr) (data not shown). At 4 hr both the TR concentration and cyGPX activity fell to below the control levels, but the differences were not significant. At 10, 24 and 48 hr the cyGPX activity was elevated above the control levels, but the differences were not significant due to very large variations in the data.

3.3.17 The potential of endothelial cells to oxidise native LDL, and the effect of Se supplementation on the oxidation process

a) EAhy926 cells

Figure 3.32 shows the effect of sodium selenite supplementation (of EAhy926 cells) and/or $CuSO_4$ supplementation (of LDL) on the relative electrophoretic mobility (REM) of LDL. Figure 3.32a demonstrates that the EAhy926 cells did not alter the REM of the LDL compared to the LDL without cells present (lanes 7 and 3). When the EAhy926 cells were in differing culture media, oxidation of the LDL differed. The REM of the LDL when incubated with EAhy926 cells in Ham's F-12 medium (lane 5) was higher than that when the cells were cultured in DMEM (lane 3). The Se status of the cells did not attenuate the slight alteration in REM seen in cells cultured in Ham's F-12 medium (lanes 5 and 6). A slight increase in REM of LDL in the absence of cells was demonstrated when the LDL was treated with 2.5 μM Cu, also in the absence of cells (lanes 7 and 8).

Figure 3.32b again demonstrates the increased REM of LDL treated with Cu in the absence of cells; 5 μM Cu increased the REM of the LDL (lane 5) to a greater extent than 2.5 μM Cu (lane 4) in comparison with LDL not treated with Cu (lane 3). The LDL in the absence of cells had a very slight increase in REM when diluted in Ham's F-12 medium (lane 6) in comparison with dilution in DMEM (lane 3). When cells were present, the REM of the LDL diluted in Ham's F-12 was slightly increased (lane 7) over that seen when cells were absent (lane 6).

Four gels were run in total for EAhy926 cells, but only two are shown here as representative results.

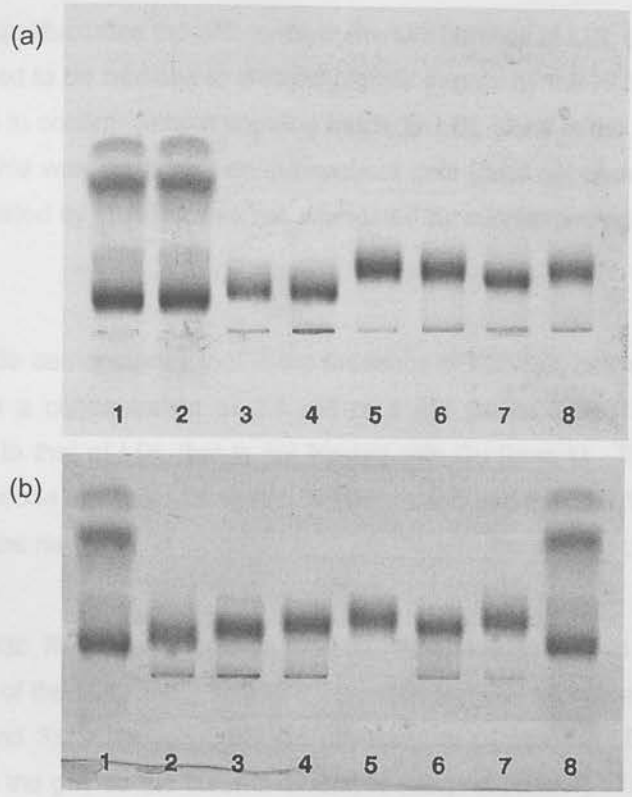


Figure 3.32 Lipoprotein gel of native low density lipoprotein (LDL) incubated with EAhy926 cells for 24 hr. Cells received Se-deficient medium containing either 40nM sodium selenite, or unsupplemented DMEM medium for a 48 hr pre-incubation. The cells were then washed, and received native LDL, supplemented with 2.5 μM or 5 μM $CuSO_4$, or unsupplemented, diluted in serum-free DMEM medium or Ham's F-12 medium (200 μg/ml final concentration). Incubation with LDL was for 24 hr. Upon completion of the incubation, the LDL was removed from the wells and BHT (25 μM) and EDTA (100 μM) added to arrest further oxidation. Normal serum was included to serve as a standard.

(a) Lane 1, normal serum with no additions; lane 2, normal serum with no additions; lane 3, Se-deficient cells with LDL in DMEM; lane 4, Se-supplemented cells with LDL in DMEM; lane 5, Se- deficient cells with LDL in Ham's F-12; lane 6, Se- supplemented cells with LDL in Ham's F-12; lane 7, LDL in DMEM without cells; lane 8, LDL supplemented with 2.5 μM Cu in DMEM without cells.

(b) Lane 1, normal serum with no additions; lane 2, Se-deficient cells with LDL in DMEM; lane 3, LDL in DMEM without cells; lane 4, LDL supplemented with 2.5 μM Cu in DMEM without cells; lane 5, LDL supplemented with 5 μM Cu in DMEM without cells; lane 6, LDL in Ham's F-12 without cells; lane 7, Se-deficient cells with LDL in Ham's F-12; lane 8, normal serum with no additions.

b) HUVEC

Figure 3.33a illustrates the difference in the two batches of LDL (lanes 2 and 4) in that batch 'B' appeared to be oxidised to a slightly larger degree by the HUVEC than batch 'A'. This is impossible to confirm without showing batch 'B' LDL alone in the absence of cells to act as a control. This was confirmed on subsequent gels (data not shown). The small increase in REM mediated by HUVEC was not attenuated by supplementing the HUVEC with Se (lanes 2 and 3).

Figure 3.33b demonstrates that in the presence of HUVEC, native LDL that is supplemented with Cu at a concentration of 2.5 μM or 5 μM (lanes 2 and 3) has an increased REM compared to that of LDL that is not treated with Cu (lane 1). This was also demonstrated with the second batch of LDL (batch 'B') (lanes 4, 5 and 6). The Se status of the cells did not attenuate the rise in REM mediated by 5 μM Cu with the second LDL batch (lanes 6 and 7).

Figure 3.33c illustrates oxidation of LDL mediated by Cu in the absence of HUVEC. Treatment of the LDL with 2.5 μM Cu increased its REM compared to LDL untreated with Cu (lanes 2 and 3). Although 5 μM Cu appeared to oxidise the LDL, the sample did not run straight on the gel, so the band is difficult to interpret (lane 4). This was confirmed on other gels subsequently (data not shown).

Five gels were run in total for HUVEC, but only three are shown here as representative results.

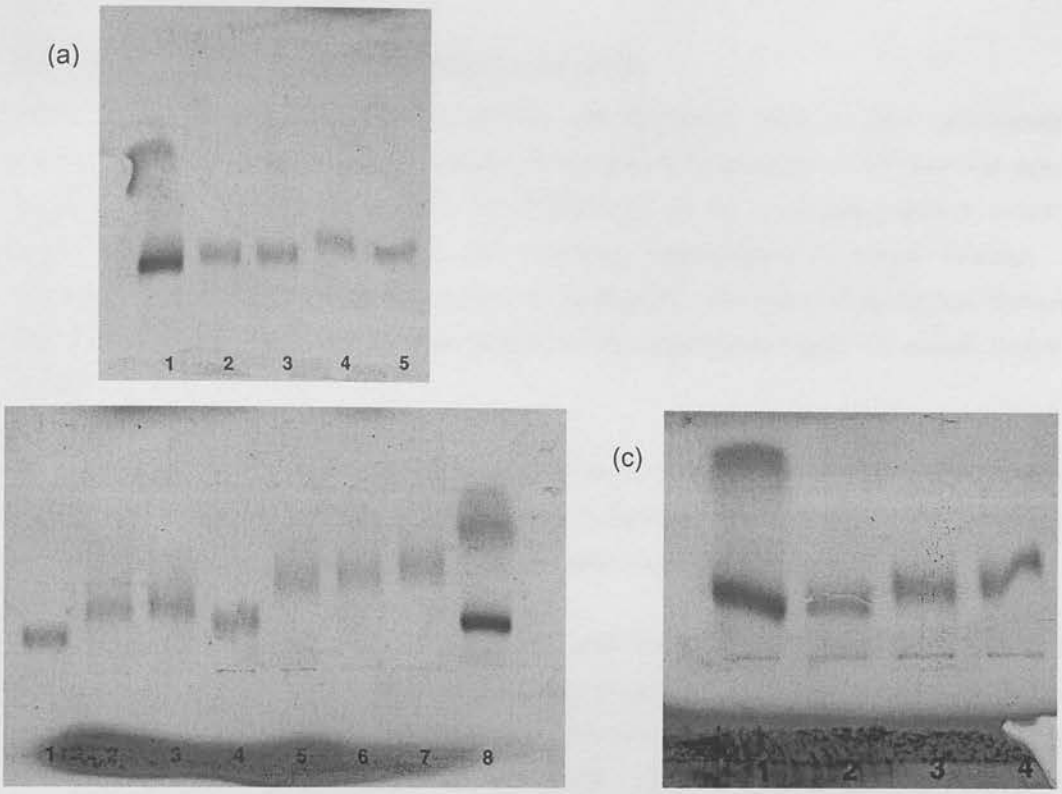


Figure 3.33 Lipoprotein gel of native low density lipoprotein (LDL) incubated with HUVEC for 24 hr. Cells received Se-deficient medium containing either 40nM sodium selenite, or unsupplemented M199 medium for a 48 hr pre-incubation. The cells were then washed, and received native LDL, supplemented with 2.5 μM or 5 μM $CuSO_4$, or unsupplemented, diluted in serum-free M199 (200 μg/ml final concentration). Incubation with LDL was for 24 hr. Two different LDL preparations were used ('A' and 'B') in parallel. Upon completion of the incubation, the LDL was removed from the wells and BHT (25 μM) and EDTA (100 μM) added to arrest further oxidation. Normal serum was used as a standard.

(a) Lane 1, normal serum with no additions; lane 2, Se-deficient HUVEC with native LDL batch 'A'; lane 3, Se-supplemented HUVEC with native LDL batch 'A'; lane 4, Se-deficient HUVEC with native LDL batch 'B'; lane 5, Se-supplemented HUVEC with native LDL batch 'B'.

(b) Lane 1, Se-deficient HUVEC with native LDL batch 'A'; lane 2, Se-deficient HUVEC with LDL batch 'A' supplemented with 2.5 μM Cu; lane 3, Se-deficient HUVEC with LDL batch 'A' supplemented with 5 μM Cu; lane 4, Se-deficient HUVEC with native LDL batch 'B'; lane 5, Se-deficient HUVEC with LDL batch 'B' supplemented with 2.5 μM Cu; lane 6, Se-supplemented HUVEC with LDL batch 'B' supplemented with 5 μM Cu; lane 7, Se-supplemented HUVEC with LDL batch 'B' supplemented with 5 μM Cu; lane 8, normal serum with no additions.

(c) Lane 1, normal serum with no additions; lane 2, native LDL batch 'A' without cells; lane 3, LDL (batch 'A') supplemented with 2.5 μM Cu, without cells; lane 4, LDL (batch 'A') supplemented with 5 μM Cu, without cells.

3.4 DISCUSSION

Model systems of human endothelial cells

BAEC differed considerably from HUVEC and EAhy926 cells in their selenoprotein expression, particularly in their TR activity which was 76 % lower ($p < 0.05$) than the activity found in HUVEC. These data imply that BAEC may not be a suitable model in which to study selenoproteins in relation to atherosclerotic mechanisms in human disease. A difference in the ability of sodium selenite to protect EC has been demonstrated between BAEC and HUVEC, and this may be related to the significantly lower TR activity found in BAEC.

There were no differences in the expression of TR, cyGPX or PHGPX between HUVEC and HCAEC. The similar selenoprotein expression in these two cell types was also reflected in the similar ability of sodium selenite to protect against oxidative damage by t-BuOOH.

The endothelial cell line EAhy926 has been used for a number of studies of endothelial function. The cyGPX activity in EAhy926 cells was only 27 % of that found in HUVEC ($p < 0.0001$), but PHGPX activity in the EAhy926 cells was no different to that found in HUVEC. The basal medium for EAhy926 cells is different from that used to culture HUVEC, and HCAEC, and contains a different Se concentration. For a true comparison to be made, all the cell types would need to be cultured in the same medium, but this was not possible. Although the basal cyGPX activity was different in EAhy926 cells and HUVEC, the selenoenzyme was induced to a similar amount in both cell types upon supplementation with similar selenite concentrations. TR was the dominantly expressed selenoprotein in both HUVEC and EAhy926 cells (figure 3.01), and the TR activity and concentration were not significantly different between cell types (figure 3.02). Furthermore, the sensitivity of EAhy926 cells to t-BuOOH and the concentrations of selenite that confer optimal protection from t-BuOOH toxicity are also similar to previous results using HUVEC and HCAEC. These data taken together indicate that the EAhy926 cell line may be a suitable and convenient model to study the role of Se in preventing oxidative damage to human EC.

The influence of variations in confluence and FCS on cytotoxicity

The susceptibility of EAhy926 cells to damage by t-BuOOH was variable in early experiments. This variability was related to the degree of confluence and FCS concentration, with the most susceptible cells being those with a low degree of confluence or low FCS concentration.

Alterations in signalling pathways accompany cellular confluency (Rizzino *et al.*, 1988), and rapidly dividing cells may produce more ROS, thereby changing cellular signalling and redox

state. Growth arrest induced by contact inhibition is partly due to a decrease in steady state levels of ROS with consequent impairment of redox signalling (Pani *et al.*, 2000). These alterations in signal transduction pathways may affect the antioxidant status of the cell, rendering it less susceptible to cytotoxic insult. Certain cell lines which exhibit density limitation of growth demonstrate induction of Mn-SOD upon cessation of proliferation of cells in culture (Oberley *et al.*, 1995). SOD activities also vary with the phases of the cell cycle (Oberley *et al.*, 1995). The toxicity of oxLDL to fibroblasts (Kosugi *et al.*, 1987) and UVB to keratinocytes (Horio and Okamoto, 1987) varies with the stage of cell cycle.

Cellular energy consumption may be dominated by cell replication at low cell densities, whereas cellular energy could be redirected to differentiated functions at higher cell densities (Krekels *et al.*, 1991). Cytotoxicity of LDL to EC is greater in subconfluent cells than confluent cells (Hessler *et al.*, 1979; Hodis *et al.*, 1994; Kosugi *et al.*, 1987).

Antioxidant enzymes may vary between different stages of confluency in some cell types (Bishop *et al.*, 1985), but our experiments did not show any significantly reproducible changes in TR, cyGPX, or PHGPX activity in EAhy926 cells with differing confluence levels (data not shown). This does not, of course, rule out differences in antioxidant enzymes other than the selenoenzymes studied here.

The cytotoxicity of t-BuOOH to EAhy926 cells was inversely proportional to the concentration of FCS in the medium, either in which the cells were growing or in which the t-BuOOH was dissolved prior to addition to the cells. FCS attenuates *in vitro* cytotoxicity of silver nitrate to human dermal fibroblasts (Hidalgo and Domínguez, 1998), β-carotene cytotoxicity to human colonic tumour cells (Peram *et al.*, 1996), and organochlorine cytotoxicity to human placental choriocarcinoma cell lines (Letcher *et al.*, 1999). The serum content of culture medium can directly decrease ROS generation (Bishop *et al.*, 1985). Hydrophobic compounds can be sequestered by serum proteins, such as albumin, thus decreasing the effective free concentration of toxicant accessible to the cell (Fisher *et al.*, 1993). In addition, FCS contains various growth factors, attachment factors, protease inhibitors and binding proteins which may affect the response of the cell to cytotoxic insult.

For all further experiments, the concentration of FCS and confluence level were kept constant, at 10 % FCS and full confluency for treatment of the EAhy926 cells.

Protection against t-BuOOH- and oxLDL-mediated cytotoxicity in EAhy926 cells by selenium

As in HUVEC and HCAEC, oxidative damage to EAhy926 cells by t-BuOOH could be prevented by pre-incubation with low nanomolar concentrations of selenite. Such protection was also afforded to EAhy926 cells against cytotoxicity mediated by oxLDL. Protection was accompanied by significantly increased TR, cyGPX and PHGPX expression, optimal TR and cyGPX expression being achieved at concentrations ranging from 10 nM to 40 nM sodium selenite. PHGPX activity also responded to sodium selenite supplementation in a dose-dependent manner, but in both experiments high doses of selenite were required to achieve significant induction (200 nM and 1000 nM). It is likely however that the lack of effect of Se at low concentrations was due in part to the poor precision of the PHGPX assay at the low activities being measured in the cells.

Plasma Se levels have been measured for 15 to 18 year old males and females in the UK, giving values of 0.89 +/- 0.14 μmol/L (n = 159) for males, and 0.91 +/- 0.14 μmol/L (n = 163) for females (Bates *et al.*, 2002a). The Se level in plasma of UK residents over the age of 65 years is similar to those measured for 15 to 18 year olds, at 0.9 μmol/L (n = 1134) (Bates *et al.*, 2002b). Selenium deficiency is defined as a plasma concentration at or below 85 μg/L, a concentration not attained in many northern European countries (Rayman, 2000). Such *in vivo* Se concentrations (μM) are dramatically higher than the concentrations required to protect endothelial cells and skin cells *in vitro* from oxidative stress, and to maximally upregulate TR, cyGPX and PHGPX (nM). This apparent discrepancy may arise due to albumin, selenium-binding proteins, and SelP taking up Se in the plasma, meaning that a higher concentration is required to have the effects seen with lower concentrations *in vitro*. In addition, thiols in the plasma may react with sodium selenite, lowering the amount of Se that is bioavailable for selenoprotein synthesis. Using cells *in vitro*, the whole amount of supplemental Se is available to the cells in its entirety at the same time. This is obviously not an accurate reflection of the *in vivo* situation.

Pre-incubation of EAhy926 cells with selenite, thereby inducing TR, cyGPX and PHGPX, was necessary to provide protection against t-BuOOH cytotoxicity. At sodium selenite concentrations which provided protection against t-BuOOH and oxLDL cytotoxicity in EAhy926 cells, the activity of TR, cyGPX and PHGPX were all maximally induced. To determine the relative importance of each of these selenoenzymes in the protection of EAhy926 cells against oxidative stress, a selective gold thioglucose (GTG) concentration was established to selectively inhibit TR.

A 48 hr pre-incubation with GTG (1 μM) resulted in 74.8 % of TR activity retention compared to control EAhy926 cells ($p < 0.01$), whilst cyGPX and PHGPX activities were not significantly changed (figure 3.21). EAhy926 cells treated in this way were significantly more susceptible to cytotoxicity from t-BuOOH ($p < 0.05$) (figures 3.24, 3.26, 3.28) and oxLDL ($p < 0.05$) (figure 3.31 a). These data suggest that factors other than GPX, perhaps TR, may play an important role in preventing damage to human endothelial cells from oxidised lipids. The fact that such a small inhibition of TR activity by 1 μM GTG produced a significant increase in susceptibility of the cells to damage by t-BuOOH or oxLDL suggests that although TR is present in high concentrations in EC, such high concentrations are required for optimal protection from oxidative stress. Alternatively, it could be that this concentration of GTG has inhibited an as yet unidentified protein/enzyme that is important in cytoprotection (see below).

Cells treated with GTG at a concentration that inhibited both TR and the GPXs (10 μM) (14.0 % TR activity retention; $p < 0.001$, 40.2 % cyGPX activity retention; $p < 0.001$, 77.5 % PHGPX activity retention; $p < 0.01$) were more susceptible to t-BuOOH toxicity than cells in which only TR was inhibited (1 μM GTG). This may suggest that under normal circumstances both TR and the GPXs may be involved in prevention of oxidative damage to human endothelial cells. However, it could also be that the increased susceptibility of the cells to damage when treated with 10 μM is due, alone or partly, to a greater level of inhibition of TR activity alone. Selective inhibition of the GPXs in future studies, with specific inhibitory antibodies for example, would help to further elucidate the role of the GPXs in an antioxidant role in endothelial cells.

The protective effects of Se supplementation were able to override the deleterious effects of GTG treatment prior to t-BuOOH or oxLDL exposure. These studies further suggest that both TR and the GPX selenoenzymes are important in protection from the oxidative damage produced by oxidised lipids. This reinforces the importance of an adequate Se status in cells which may have impaired antioxidant status due to oxidative stress as a result of disease states or pharmacological interventions.

The differing sensitivities of the selenoenzymes TR and GPX to inhibition by gold compounds have been shown both *in-vitro* (Gromer *et al.*, 1998) and *in-vivo* (Smith *et al.*, 1999). The GPXs are relatively resistant to inhibition whilst TR is very sensitive, having an IC_{50} ~ 1000-fold lower than that of the GPXs (Gromer *et al.*, 1998). The differences in the susceptibility of the selenoenzymes to inhibition by GTG was also shown in human placental cytosol, where GTG concentrations of 150 μM and 96 nM GTG were required to inhibit 50 % of cyGPX activity and TR activity respectively. This ~1500-fold difference is comparable to data reported by Gromer *et al.* In endothelial cells, a concentration of 1 μM GTG

produced 74.8 % retention of TR activity ($p < 0.01$), but the same concentration produced < 1 % retention of TR activity in human placental cytosol. This may imply that the cells contain other molecules in their cytosol which react with the GTG, thus requiring a higher concentration to inhibit selenoenzymes. Alternatively, the cells may actively take up the GTG and concentrate the compound.

These data cannot exclude the possibility that GTG has inhibited another, as yet, unidentified selenoenzyme with an antioxidant role. Gold compounds may have other effects in the cells which could affect their response to oxidative insult, including induction of anti-oxidative genes including haem oxygenase-1 and γ-glutamylcysteine synthetase (Kataoka *et al.*, 2001), down-regulation of the expression of genes involved in the inflammatory response (collagenases, cytokines (interleukins, and TNF-α) and producers of chemical mediators (cyclooxygenase-2 and iNOS)) (Yoshida *et al.*, 1999) (Yamashita *et al.*, 1999) (Bondesona and Sundlera, 1995) , and inhibition of the DNA-binding activity of NFκB and AP-1, that regulate a range of genes (Handel *et al.*, 1995). Gold compounds also inhibit proliferation of HUVEC, suggesting an antiangiogenic effect (Matsubara and Ziff, 1987), and may inhibit PKC *in vitro* (Mahoney *et al.*, 1989), altering cellular signalling pathways and concomitant cellular functions.

The cytotoxicity of oxLDL to EAhy926 cells and HUVEC, and activities of SOD, catalase and cyGPX have previously been compared (Claise *et al.*, 1997). EAhy926 cells had significantly lower SOD, catalase and cyGPX activities compared with HUVEC, and were susceptible to damage by 150 μg/ml oxLDL. No cytotoxicity was observed in HUVEC using oxLDL concentrations of up to 200 μg/ml (Claise *et al.*, 1997). SOD and CAT were ineffective in protecting cells from oxLDL cytotoxicity in both cell types (Claise *et al.*, 1997), whilst pre-incubation of EAhy926 cells with cyGPX protected against oxLDL cytotoxicity. The higher susceptibility of EAhy926 cells to oxLDL cytotoxicity was attributed to the lower antioxidant defences of these cells compared to HUVEC, particularly with respect to the cyGPX activity which was 8 % of that of HUVEC. Our studies found the cyGPX activity in EAhy926 cells was 26 % of that found in HUVEC. The TR activity and mass and PHGPX activity were shown not to be significantly different. Claise's group did not measure the activities of either TR or PHGPX.

At the concentration of oxLDL at which cytotoxicity was first evident to the EAhy926 cells, total glutathione was depleted to below the lower detection limit of our assay. Whilst glutathione is important in preventing oxLDL-induced damage (Moellering *et al.*, 2002) (Cho *et al.*, 1999), the doses of Se and GTG used in our experiments produced no significant modification to intracellular glutathione concentrations. It is likely that the fall in GSH levels

was due to leakage from cell membranes damaged by oxLDL toxicity. In EAhy926 cells oxidised lipids from cholesteryl esters induce GSH depletion without inducing cytotoxicity, whilst fractions containing oxysterols were more cytotoxic than GSH-depleting, suggesting that their cytotoxicity is mediated by a GSH-independent mechanism (Thérond *et al.*, 2000). TR may be a candidate for such GSH-independent effects, whilst GSH-dependent effects could be due to GPX, glutathione-S-transferase, or direct interaction.

Cell injury induced by oxLDL may be due to lipid peroxidation involving lipid hydroperoxide-induced, iron-mediated formation of alkoxyl, lipid and peroxyl radicals (Coffey *et al.*, 1995). The Se mimic Ebselen has cytoprotective effects against oxLDL toxicity (Coffey *et al.*, 1995). Detoxification of LOOH is through a GPX-mediated reduction. However, the TR /Trx system has a high capacity to detoxify LOOH and may provide an important alternative to the GPXs for detoxification (Björnstedt *et al.*, 1995), as suggested by our data.

Studies on oxLDL are complicated by the varying chemical composition of oxLDL from different pools (Esterbauer and Jürgens, 1993). There are distinct variations in the degree to which LDL preparations from different donor individuals can be modified (van Hinsberg *et al.*, 1986). Foetal calf serum (FCS) was not excluded from the culture media for our oxLDL toxicity studies, but some studies report excluding it or replacing it for the duration of the incubation with oxLDL. FCS may have a buffering effect which may provide some cytoprotection. We have observed differing susceptibilities in EC to t-BuOOH-mediated cytotoxicity depending on the level of FCS in the medium (see above sections). The FCS may contain trace amounts of ceruloplasmin which may contribute to further lipid peroxidation, depending on the culture medium used (Burkitt, 2001).

The data of Avogaro *et al.* (Avogaro *et al.*, 1991) quotes normal LDL levels in middle-aged men of 3.7 (2.3 – 5.2) mMol/L (mean (5 and 95% CI)). Since LDL contains 25 % protein and 75 % lipid, it is possible to make an estimate of an *in vivo* oxLDL concentration of 31.5 mg/L. Although similar oxLDL concentrations to this were used in the cytotoxicity experiments in this thesis, such concentrations were not cytotoxic. The higher concentrations (maximum 1600 mg/L) of oxLDL were cytotoxic to EAhy926 cells, but are obviously much higher than physiological levels of oxLDL seen *in vivo*.

Modulation of oxidation of native LDL by selenium supplementation of endothelial cells

In vitro studies suggest the formation of oxLDL is mediated in part by the enhanced production of ROS by the cell types which constitute the arterial wall. The data presented provides no evidence to agree with previous studies that have shown HUVEC to oxidise LDL

to a degree easily detected by lipoprotein electrophoresis (Fernando *et al.*, 1998). Differing media were used to culture the HUVEC in our studies (Medium M199) compared to those of Fernando *et al.* (RPMI medium), which differ in their components, including amino acids and vitamins. This may explain some of the disparity in results. Chelating agents which interfere with the Cu^{2+} binding to the LDL particle, including EDTA, free histidine and other components of cell culture media, can protect the particle from oxidation (Burkitt, 2001). Other substances in culture media which vary between media and may affect the oxidation of LDL by EC include phenol red and ascorbic acid (Steinbrecher, 1988). Amino acids and vitamins in buffer ameliorate ROS-mediated chlorobenzene hepatotoxicity *in vitro* (Fisher *et al.*, 1993).

When native LDL was added to EAhy926 cells or HUVEC for an incubation of 24 hr, oxidation of the LDL by the cells was dependent upon transition metal ions in the culture medium, confirming previous observations (Burkitt, 2001) (Steinbrecher *et al.*, 1984) (Steinbrecher, 1988). Oxidation of LDL took place to a similar degree when copper was present in the absence of cells. Oxidation of LDL by Cu in the absence of cells is a well-reported phenomenon (Burkitt, 2001; Heinecke, 1998; Lamb and Leake, 1992; Steinbrecher *et al.*, 1984). It is unsurprising that Se supplementation of EAhy926 cells and HUVEC did not alter their oxidation of native LDL since the cells did not oxidise LDL in the absence of Se supplementation.

Conclusions

Whilst Se has been shown to protect against t-BuOOH-mediated cytotoxicity in HCAEC and HUVEC, there is insufficient existing evidence regarding the relative importance of TR and the GPXs in the role of the protection. The data presented here suggest that Se can protect human EC against t-BuOOH-and oxLDL-mediated cytotoxicity *in vitro*. Furthermore, the use of GTG in our study has allowed us to begin to associate some of the effects of Se with specific selenoproteins, and in particular suggest that both TR and the GPXs are involved in antioxidant protection of the endothelium. These multiple enzyme systems may act in different cellular compartments.

Our observations support the view that a low Se status would promote endothelial injury and atherosclerosis initiated by oxLDL in humans. The results presented suggest that Se supplementation at doses which optimise the expression of the TR and the GPXs may have significant beneficial effects when applied to populations that have a Se intake below that currently recommended.

CHAPTER FOUR

THE ROLE OF THIOREDOXIN REDUCTASE IN THE PREVENTION OF OXIDATIVE DAMAGE TO THE SKIN BY SELENIUM

4.1 INTRODUCTION

4.1.1 UVB and the skin

The UK population is increasingly being exposed to ultraviolet (UV) irradiation. Such exposure produces photo-oxidative damage induced by ROS, leading to phototoxicity, photoaging and skin cancer. UVB radiation can damage cells by numerous means and is far more effective at causing cell death than UVA. Exposure to UVB can induce DNA damage, protein cross-linking and the production of free radicals and ROS, which can damage DNA and induce lipid peroxidation in cell membrane lipids (Danno *et al.*, 1984; Horio and Okamoto, 1987; Miyachi *et al.*, 1983). Further effects of UVB radiation are discussed in detail in chapter 1. *In vitro*, cultured cells exposed to high doses of UVB radiation exhibit necrotic cell death. However at lower doses of UVB radiation cultured cells are reported to undergo apoptosis (Mammone *et al.*, 2000). Damaged keratinocytes known as sunburn cells are characteristic of UVB- and UVC-induced epidermal damage (Soter and Baden, 1991), and are thought to be undergoing apoptotic cell death (Bayerl *et al.*, 1995). Melanocytes, keratinocytes and fibroblasts have all demonstrated susceptibility to cell death mediated by UVB radiation (Dissanayake *et al.*, 1993).

4.1.2 Skin cells and oxidative stress

Defence strategies of the skin against endogenous and exogenous ROS include small radical trapping molecules, such as vitamins A, C and E, thiols, such as reduced glutathione, and enzymes such as SODs, catalase, GPX, TR/Trx and the thioredoxin peroxidases. There are also intrinsic free radical traps in melanin. UV exposure of human and animal skin produces a range of ROS which may derive from melanin, from reactions with photosensitizers (e.g. phenothiazines, cosmetics, food additives), or may be part of the inflammatory response of erythema (Fuchs, 1998; Maccarrone *et al.*, 1997; Schallreuter and Wood, 1989). Cellular sources of ROS, and ROS generation in the skin are described in detail in section 1.8.

4.1.3 Selenium and the skin

The upregulation of selenoprotein expression in cultured cells through Se-supplementation has been extensively reported (Buckman *et al.*, 1993; Dreher *et al.*, 1998; Ricetti *et al.*, 1994; Takahashi *et al.*, 1986; Thomas *et al.*, 1993; Yarimizu *et al.*, 2000), including skin cells (Marcocci *et al.*, 1997; Stewart *et al.*, 1999). The function and regulation of the GPX and TR selenoenzymes have been discussed in detail in Chapter 1. In conditions of Se deficiency, decreased expression of GPX and TR could increase the susceptibility of cells to ROS-induced damage. TR has been reported to be located on the surface of keratinocytes, an ideal location to detoxify ROS (Schallreuter and Wood, 1989). Immunohistochemical staining for TR and Trx in skin of adult rats has shown localisation in the stratum germinativum, hair follicles, nail beds and sweat glands (Rozell *et al.*, 1985). Keratinizing cells stained positive for Trx only, whilst melanocytes showed staining for TR and Trx. A study by Lee *et al.* has confirmed the expression of TR and Trx to be mainly in the hair follicle in rat skin, with only weak staining of both proteins in the basal cell layer of the epidermis (Lee *et al.*, 2000).

There is abundant evidence to suggest that Se has an important role in protecting skin from the deleterious effects of UVB exposure. Low plasma Se levels have been inversely linked to skin cancer (Clark *et al.*, 1984; Reinhold *et al.*, 1989), and plasma Se status is reported to be predictive of future skin cancer risk (Combs *et al.*, 1993).

Supplementation of human skin fibroblasts with sodium selenite (1.2 μM) decreased cell death from 50 % to 25 %, following exposure to 120 J/m^2 UVB (Richard *et al.*, 1990). Similar results using UVA radiation and sodium selenite (1.3 μM) reported that cell death in human skin fibroblasts was decreased from 50 % to 10 % (Leccia *et al.*, 1993). However, these studies used Se concentrations that are not achievable *in vivo*. In a subsequent study using more physiological Se concentrations, supplementation with 320 nM sodium selenite decreased cell death of human skin fibroblasts from 67 % to 30 % following exposure to UVA (Moysan *et al.*, 1995b). Pre-treatment of primary keratinocytes, melanocytes, or HaCaT cells with sodium selenite or selenomethionine protects each of these cell types from UVB-induced cell death (Rafferty *et al.*, 1998). Sodium selenite provided optimum protection at 10 nM in all cell types tested, whereas with selenomethionine concentrations of 50 nM, 100 nM and 100 nM were required for optimal protection of keratinocytes, HaCaT cells and melanocytes respectively (Rafferty *et al.*, 1998). Se protects DNA from oxidative damage, but not from direct damage by UVB irradiation (Rafferty, 2000).

In 1965 Shamberger and Rudolph demonstrated a significant reduction of skin cancer incidence in carcinogen-treated mice given a topical application of sodium selenite

(Shamberger and Rudolf, 1965). In later studies, Se supplementation decreased the incidence of skin tumours in mice (Overvad *et al.*, 1985; Pence *et al.*, 1994), and inflammation and sunburn in hairless mice following exposure to UVB (Thorling *et al.*, 1983). Se, given both orally and topically, increases the minimal erythemal dose (MED) in humans (Burke *et al.*, 1992a; Burke *et al.*, 1992b), and decreases the number of sunburn cells in human skin following UVB exposure (la Ruche and Césarini, 1991). It should be noted that in two of the murine studies which showed protection with Se (Overvad *et al.*, 1985; Thorling *et al.*, 1983) the doses of Se were so high (reaching 8 mg/L) (Overvad *et al.*, 1985) as to be unacceptable for human use.

Polyunsaturated fatty acids (PUFA) in the phospholipids of plasma membranes are one of the cellular targets of ROS. Malondialdehyde (MDA) is one of the main aldehyde by-products of lipid peroxidation. The thiobarbituric acid (TBA) assay is the most commonly used method to assess lipid peroxidation, and is based upon the reaction of TBA with MDA to form an adduct. The term 'thiobarbituric acid reactive substances' (TBARS) is often used to describe the results more accurately since other substances can also react with TBA such as other lipid peroxides, amino acids and sugars.

MDA is formed, in a dose-dependant manner, following exposure of cells to UVB (Stewart *et al.*, 1996). Exposure to UVA also causes MDA formation in a dose-dependent manner (Moysan *et al.*, 1995b). Se also decreases the formation of TBARS in humans *in vivo*. When given prior to exposure of patients to a solar simulator, Se (200 μg/day) decreased TBARS formation by 13 % (Pietschmann *et al.*, 1992). Furthermore, cultured fibroblasts supplemented with sodium selenite (320 nM) for 3 days prior to exposure to UVA exhibited a 50 % decrease in the formation of TBARS (Leccia *et al.*, 1993; Moysan *et al.*, 1995b).

4.1.4 Antioxidant supplementation and the skin

Other antioxidants apart from selenoenzymes, such as vitamin C (ascorbate), α-tocopherol and glutathione, can protect human skin cells *in vitro* from UVB-induced cell death, apoptosis and MDA formation (Fuchs, 1998; Kondo *et al.*, 1990; Savini *et al.*, 1999; Straface *et al.*, 1995). These results are strongly indicative that UVB exposure produces ROS in the skin. Antioxidants such as selenoproteins can prevent ROS-induced damage to skin cells by detoxifying these molecules.

4.1.5 Methods to assess cytotoxicity

Interpretation of the significance of assay results is dependent upon distinguishing between assays which measure cytotoxicity and those measuring cell survival. When the oxidative stressor exhibits toxicity which is not specifically related to proliferative potential, and results

in loss of essential cell functions rather than loss of reproductive capacity, a cytotoxicity test is appropriate. Cytotoxicity assays measure oxidative stress-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death, whereas survival assays measure the end result of such metabolic perturbations which may be either cell recovery or cell death.

Two different assays have been utilised in this chapter to assess cytotoxicity. The trypan blue assay was employed to measure cell viability following UVB irradiation, whilst lactate dehydrogenase (LDH) activity retention was used to measure viability following menadione treatment in HaCaT cells. LDH can still leak from viable cells, so LDH retention is actually an assessment of membrane integrity rather than true viability. The trypan blue dye exclusion method relies on the fact that live cells exclude dye, whilst dead cells take up the dye, thereby also relying on membrane integrity. Membrane leakiness can also be caused by recent trypsinisation and freezing/thawing in the presence of DMSO, so care has to be taken with cell cultures to avoid artifactual membrane damage. A decrease in cell number has been reported during trypsinisation of cells which have been UVA-damaged, due to the cells disintegrating (Moysan *et al.*, 1995a), further reinforcing the importance of avoiding damage to already-injured cells.

4.1.6 Skin cells used in UV studies

Keratinocytes are the most numerous cell type in the epidermis, receive the greatest exposure to UVB, and are the cell type that forms basal and squamous cell carcinomas. Basal and squamous cell carcinomas are likely to be induced by exposure to UVB radiation (Black *et al.*, 1997; Stenbäck, 1975; Urbach, 1997). The majority of experiments in this chapter were performed using the human keratinocyte cell line HaCaT, while a limited number of studies have been performed on primary keratinocytes due to lack of available material for cell preparations. Established epidermal and dermal cell lines are frequently used in studies of UV-induced cellular damage *in vitro*. The keratinocyte cell line HaCaT is a spontaneously transformed human epithelial cell line from adult skin that maintains full epidermal differentiation capacity (Boukamp *et al.*, 1988). This cell line has been used in numerous studies (Aragane *et al.*, 1998; Didier *et al.*, 1999; Göhring *et al.*, 2000; Haycock *et al.*, 2000; Kroll *et al.*, 1999; Leccia *et al.*, 1998; Lehmann *et al.*, 1998; Licht *et al.*, 1992; Mammone *et al.*, 2000; Marcocci *et al.*, 1997; Petersen *et al.*, 2000; Podhaisky *et al.*, 2000; Saliou *et al.*, 1999; Savini *et al.*, 1999; Savini *et al.*, 2000; Schürer *et al.*, 1993). However, due to mutations on both p53 alleles (p53 -/-) (Magal *et al.*, 1998), the HaCaT cell line may not behave exactly like native keratinocytes (Göhring *et al.*, 2000; Merryman, 1999).

A study by Leccia *et al.* has demonstrated that HaCaT cells are more resistant to UVA irradiation than normal human primary keratinocytes (Leccia *et al.*, 1998), and found that the cell line contains lower GPX and SOD activities, but higher total GSH than primary keratinocytes. The sensitivity of primary and cell lines cannot easily be explained by variations in antioxidant profiles alone, and may involve media composition, number of subcultures, or confluency (Leccia *et al.*, 1998). The sensitivity of keratinocytes to UV irradiation is also related to the cell cycle (Horio and Okamoto, 1987).

4.1.7 Model agents as oxidative stressors

Menadione, a synthetic vitamin K, is used frequently as a model toxicant (Buckman *et al.*, 1993; Cho *et al.*, 1997; Comporti, 1989; Malorni *et al.*, 1993; Rosen and Freeman, 1984; Santini *et al.*, 1996). Menadione (2-methyl-1,4-naphthoquinone) is a strong oxidising, redox-cycling agent that can generate a great quantity of ROS when it enters cells (Thor *et al.*, 1982). These ROS are produced by one-electron reduction of quinones to semiquinone radicals, which can rapidly reduce dioxygen to form $O_2^{\bullet-}$, and subsequently H_2O_2, $OH^{\bullet}$, and singlet oxygen by dismutation of $O_2^{\bullet-}$ (Comporti, 1989). When the rate of redox cycling of menadione exceeds the capacity of antioxidant enzymes, cytotoxicity occurs.

4.1.8 Inhibitors of TR

Several *in vitro* inhibitors of TR have been reported (Becker *et al.*, 2001; Lin *et al.*, 1999; Lin *et al.*, 2001; Schallreuter and Wood, 1987). The clinically used inhibitors of TR (nitrosoureas of the carmustine type) only inhibit the reduced form of the enzyme (Arscott *et al.*, 1997; Williams *et al.*, 2000); the oxidized form of TR is not inactivated. Using a bioassay for TR based on reduction of a spin-labelled nitroxide, Schallreuter *et al.* have investigated the inhibition of TR by various compounds. The reduction of their spin label on skin, keratinocytes, melanocytes and purified *E. coli* TR was inhibited by thioprotein inhibitors, $NADP^+$, anthralin, azelaic acid and other saturated dicarboxylic acids, and Trx (a competitive substrate) (Schallreuter *et al.*, 1986; Schallreuter and Wood, 1987).

4.1.9 Different chemical forms of selenium

Selenomethionine is a less-available metabolic source of Se than selenite or selenate, since these need only be reduced to selenide to provide selenophosphate, the precursor of selenocysteine, the active form of Se in selenoproteins. Studies comparing the efficacy of sodium selenite and selenomethionine in the prevention of UVB-induced oxidative damage to skin cells have been performed previously (Rafferty *et al.*, 1998). However, comparative studies of the two forms of Se on induction of selenoenzymes in human skin cells have not been carried out. Previous studies have ruled out that the protective effects of Se were mediated by alterations of growth patterns or direct antioxidant effects alone (Rafferty, 2000).

It has been suggested that the effects of Se may be acting through incorporation into selenoproteins, so studies were needed to directly confirm this assumption.

The studies reported in this chapter aimed to:

- determine the suitability of the HaCaT cell line as a suitable model in which to investigate the role of Se and selenoproteins in human keratinocytes
- examine the ability of sodium selenite supplementation to protect HaCaT cells from cytotoxicity resulting from exposure to menadione or UVB irradiation, and associate any observed protection with changes in the expression and activity of TR and the activity of cyGPX and PHGPX
- investigate whether TR, cyGPX or PHGPX were contributing to the protection of HaCaT cells from cytotoxicity of menadione or UVB irradiation by use of gold thioglucose-mediated inhibition of selenoenzyme activity
- assess potential TR inhibitors using the DTNB and insulin assay systems for TR activity

4.2 MATERIALS AND METHODS

4.2.1 General methods for cytotoxicity and selenoenzyme expression studies

In all cytotoxicity studies using skin cells, the cells were passaged into 24-well culture plates using Se-deficient medium (basal medium Se content of 3.4 ng/ml), with all test conditions in triplicate wells of confluent cells, unless otherwise stated. Following incubation in the presence of menadione, both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.13. Following irradiation, the cells were harvested for trypan blue assay as detailed in section 2.3.14. Details on the Se content of cell culture media are described in section 3.1.7.

For studies of selenoprotein expression, HaCaT cells were passaged into 225 cm^2 flasks, with all test conditions in triplicate flasks (except control cells, which were grown in quadruplicate) unless otherwise stated. Following the incubation, the cells were washed twice with 30 ml EBSS, and harvested via scraping into 50 ml EBSS. Efficiency of harvesting was determined by light microscopy. The cells were then pelleted by centrifugation at 500 *x g* for 10 min. The EBSS was aspirated, and the pellets frozen at -80°C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed and lysed by sonication (three pulses of 10 sec using a Soniprep 150 Sonicator) on ice in 0.125 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1 % Triton X-100 (peroxide- and carbonyl-free). The levels of the selenoenzymes were then determined as detailed in sections 2.3.5.1, 2.3.6, 2.3.7 and 2.3.8.

4.2.2 The effect of sodium selenite or selenomethionine supplementation on intracellular selenoprotein expression and activity in HaCaT cells

HaCaT cells were passaged into 75 cm^2 or 225 cm^2 tissue culture flasks and grown to 70 % confluence in DMEM containing 5 % FCS. The cells then received medium containing 0, 1, 10, 40, 50, 100, 200 or 1000 nM sodium selenite for an incubation of 48 hr. Following the incubation, cell detachment was checked by light microscopy, the medium was sampled for LDH assay to assess any cytotoxicity (section 2.3.13), and the cells were washed twice with 10 ml EBSS, and harvested as detailed in section 4.2.1.

For incubation with selenomethionine, the culture conditions were as described above, in 225 cm^2 tissue culture flasks, using concentrations of selenomethionine ranging from 0 to 100,000 nM (triplicate flasks for each selenomethionine concentration, and quadruplicate

flasks for the control). Harvesting of the cells and treatment of the cell pellets was as detailed above.

4.2.3 The cellular localisation of TR in HaCaT cells and primary keratinocytes

Adult human primary keratinocytes and HaCaT were grown to approximately 70 % confluence on 22 x 22 cm sterile glass coverslips in six well culture plates. Each respective culture medium was 'Se-deficient 'unless stated. The medium was aspirated from the cells, and each well washed twice with 4 ml PBS. The glass coverslips were then removed from the culture plates and fixed in acetone for 3 min. After fixing, the glass coverslips were rinsed with fresh PBS, and placed back into the wells of the original culture plates which had been filled with absolute ethanol. The cells remained preserved in this state at 4°C until immunohistochemistry took place, as described in section 2.3.19.

4.2.4 Comparison of TR and cyGPX concentration in human primary keratinocytes and HaCaT cells

Human primary keratinocytes and HaCaT cells were grown in Se-deficient medium to confluence in 6 well plates. The cells were then harvested by scraping into 4 ml PBS, and immediately assayed for TR concentration (section 2.3.6) and total protein (section 2.3.9). T75 flasks of primary keratinocytes and HaCaT cells were grown in Se-deficient medium to confluence, and assayed for cyGPX activity as detailed in section 2.3.7.

4.2.5 The effect of menadione on LDH retention in HaCaT cells cultured in selenium-deficient medium

HaCaT cells were passaged into 24 well plates with Se-deficient medium. The effect of menadione (0 – 300 μM, and 0 – 240 μM, respectively, for two separate experiments) on HaCaT cell viability was determined using confluent cells. After an 18 hr incubation in the presence of menadione, both the medium and cells were harvested and the % LDH retention was determined (section 2.3.11). Exposure of cells to menadione for 18 hr has previously been reported (Bai *et al.*, 1999; Chen and Cederbaum, 1997).

4.2.6 The effect of cellular confluence level on the susceptibility of HaCaT cells to menadione-induced oxidative damage

Preliminary experiments to investigate the effect of different concentrations of menadione on LDH activity in HaCaT cells cultured in Se-deficient medium showed great variability in the menadione concentrations required to produce cell damage. One of the factors proposed to

account for this observed variability was the level of confluence of the cell monolayer, as found in EAhy926 cells. HaCaT cells were passaged into 24 well plates and left to grow in Se-deficient medium until the required level of confluence was reached. The effect of a range of concentrations of menadione (0 – 150 μM, 0 – 140 μM, and 0 – 120 μM, respectively, for three separate experiments) on the viability of HaCaT cells at differing confluence levels was determined. After an 18 hr incubation in the presence of menadione, both the medium and cells were harvested and analysed for LDH retention (section 2.3.13). All plates of cells at differing confluence level received the same menadione solutions which had been prepared and stored at 4°C until use.

4.2.7 The effect of cellular confluence level on TR expression and activity, and cyGPX and PHGPX activity in HaCaT cells

HaCaT cells were passaged into 75 cm^2 flasks and grown to 75 %, 100 % confluence and supraconfluence (2 days post-confluence) in unsupplemented, Se-deficient medium. The time period taken to reach 75 %, 100 %, and 2-days post confluence were 3, 5, and 7 days respectively from passage. Fresh medium was put on the cells every second day as required by the respective flasks. Upon reaching the required level of confluence (assessed by eye under the light microscope), the cells were washed and harvested as described in section 4.2.1. The levels of the selenoenzymes were then determined as detailed in sections 2.3.5.1, 2.3.6, 2.3.7 and 2.3.8. Triplicate flasks were grown for each different confluence level.

4.2.8 The ability of sodium selenite supplementation to protect HaCaT cells against oxidative damage resulting from menadione exposure

To investigate the possible protective effect of sodium selenite against oxidative damage mediated by menadione, HaCaT cells were passaged into 24 well plates using Se-deficient medium. The cells were then left to grow for 48 hr. Se-deficient medium to which a range of sodium selenite concentrations (0, 1, 10, 40, 50, 100, 200, 1000 nM) had been added was then placed on the cells. After an incubation period of 48 hr, the cells were washed twice with 1 ml EBSS. Menadione was then added (80 μM) prepared in Se-deficient medium, and left to incubate with the cells for 18 hr. The control was Se-deficient medium to which no menadione had been added. After 18 hr both the medium and cells were harvested and analysed for LDH retention as described in section 2.3.13.

4.2.9 Assessment of the direct effect of sodium selenite in the protection of HaCaT cells against oxidative damage resulting from exposure to menadione

To investigate whether sodium selenite can exert a direct antioxidant effect against menadione-mediated cytotoxicity in HaCaT cells, rather than through modification of selenoprotein expression, the following approach was used. HaCaT cells were passaged into 24 well plates using Se-deficient medium. After 48 hr, some cells received Se-deficient medium supplemented with 40 nM sodium selenite, whilst other cells continued to be maintained in Se-deficient medium. After an incubation period of 48 hr, all cells were washed twice with 1 ml EBSS. The cells then received Se-deficient medium supplemented with 40 nM sodium selenite simultaneously with the addition of a range of menadione concentrations (0 – 140 μM), or the same range of concentrations of menadione made up in Se-deficient, unsupplemented medium.

Control cells received no menadione or sodium selenite supplementation. After 18 hr both the medium and cells were harvested from all the culture plates and analysed for LDH activity as described in section 2.3.13.

4.2.10 The effect of gold thioglucose on the activities of cyGPX, PHGPX, and TR of HaCaT cells

An experiment was performed to study the timecourse of inhibition of TR activity in HaCaT by 10 μM GTG. HaCaT cells were passaged into 75 cm^2 flasks and grown to 70 % confluence (to parallel the growth of cells in the corresponding toxicity experiments). The cells then received medium containing 10 μM GTG for an incubation of either 24, 48 or 72 hr (triplicate flasks for each time point). Control cells received medium unsupplemented by GTG. The cells that received an incubation of 72 hr received fresh medium supplemented with 10 μM GTG after the cells had been growing for 48 hr. Following the incubation, by which time the cells had reached confluence, the cells were washed and harvested as described in section 4.2.1. The levels of TR activity were then determined as detailed in section 2.3.5.1.

Preliminary studies were carried out prior to the menadione toxicity studies described below to optimise the GTG concentration required for selective inhibition of TR activity in HaCaT cells. The HaCaT cells were passaged into 75 cm^2 flasks and grown to 70 % confluence. The cells then received medium containing 0, 1, 10, or 100 μM GTG for an incubation of 48 hr. Following the incubation, the cells were washed and harvested as detailed in section

4.2.1. The levels of the selenoenzyme activities were then determined as detailed in sections 2.3.5.1, 2.3.7, and 2.3.8.

A further experiment using identical incubation conditions was carried out, except that the cells were cultured in 225 cm^2 flasks. The cells were treated and harvested as described above.

4.2.11 The effect of gold thioglucose on the susceptibility of HaCaT cells to oxidative damage resulting from menadione exposure

To assess the effect of pre-treatment with various GTG concentrations on the susceptibility of HaCaT cells to menadione cytotoxicity, HaCaT cells were passaged into 24 well plates using Se-deficient medium, and left to grow for 48 hr. The cells then received normal unsupplemented medium, or the same medium containing 1 μM and/or 10 μM GTG for 48 hr (these concentrations of GTG have been used in an associated experiment to investigate the effect of these GTG concentrations on the selenoenzyme activities (section 4.2.10)). After incubation, cells were washed twice with 1 ml EBSS, and fresh medium containing various concentrations of menadione (0 - 150 μM; 0 – 100 μM; 0 – 140 μM) added for an 18 hr incubation. Both the medium and cells were harvested and analysed for LDH retention as described in section 2.3.13. An experiment to investigate a wider range of concentrations of GTG was also performed. The pre-incubations were with 0, 1, 2.5, 5, 7.5 or 10 μM GTG for 48 hr.

4.2.12 The effect of consecutive sodium selenite and gold thioglucose treatment on susceptibility of HaCaT cells to oxidative damage resulting from menadione exposure

To investigate whether the protection of HaCaT by Se supplementation from menadione-mediated cytotoxicity could overcome/compensate for the deleterious effect of GTG pre-incubation, the following approach was employed. HaCaT cells were passaged into either Se-deficient medium or medium supplemented with 40 nM selenite, and incubated for 48 hr. After this time, the cells were washed twice with 1 ml EBSS, and received normal unsupplemented medium, or the same medium containing 10 μM GTG for 48 hr. When the incubation was finished, the cells were again washed, and fresh medium containing various concentrations of menadione (0 – 140 μM) added for an 18 hr incubation. Both the medium and cells were harvested and analysed for LDH activity as described in section 2.3.13.

4.2.13 The effect of consecutive sodium selenite and gold thioglucose treatment on selenoenzyme activities in HaCaT cells

To determine the effects of the pre-incubation conditions described in section 4.2.12 above (i.e. 48 hr 40 nM sodium selenite alone, 48 hr 10 μM GTG alone, or 48hr 40 nM sodium selenite followed by 48 hr 10 μM GTG), HaCaT cells were passaged into 225 cm^2 flasks. Treatment of the cells (conditions and time periods, *without* menadione addition) was as described in section 4.2.11. When the incubations were over, the cells were washed and harvested as described in section 4.2.1. The activities of TR and cyGPX were determined as detailed in sections 2.3.5.1 and 2.3.7. There was insufficient cellular material to assay for PHGPX activity.

4.2.14 Comparison of LDH retention and trypan blue exclusion as measures of damage to HaCaT cells by menadione

HaCaT were passaged into a 24 well plate and grown to confluence. The cells were then exposed to menadione (0 – 160 μM) for 18 hr. The medium was removed from the cells and spun to remove any cells or cell debris. The cell-free medium was then assayed for LDH activity (LDH release in U/L) as detailed in section 2.3.13. The cells remaining in the wells were trypsinized and added to cells removed from the medium, and assessed for cell viability by Trypan Blue assay (detailed in section 2.3.14).

4.2.15 Assessment of LDH retention as a measure of damage to HaCaT cells by UVB irradiation

HaCaT cells were passaged into 24 well plates and grown to 70 % confluence. The cells were then prepared and irradiated as detailed in section 2.3.20. Cells received an irradiation dose of 0, 480, 720, 960, or 1200 J/m^2, with each plate receiving a single dose of UVB. The medium and cell lysates were harvested for LDH analysis (as detailed in section 2.3.11) at time points of 0, 6, 12, and 24 hr respectively. This timecourse of LDH release following irradiation was repeated to extend the time period to 48 hr, and to 120 hr. In the case of UVB as the stressor agent, 48 hr is the optimal time required for the cells to die. Leaving the cells for 120 hr would obviously be an unsuitable time to leave between oxidative insult and cytotoxicity assay, and was performed in this case to illustrate the inactivation of LDH alone.

4.2.16 The ability of sodium selenite supplementation to protect HaCaT cells from cytotoxicity resulting from UVB exposure

To investigate the possible protective effect of sodium selenite against UVB-mediated damage, HaCaT cells were passaged into 24 well plates using Se-deficient medium. The

cells were then left to grow for 48 hr. Selenium-deficient medium to which a range of sodium selenite concentrations (0, 1, 10, 40, 50, 100, 200, 1000 nM) had been added was then placed on the cells. After an incubation period of 48 hr, the cells were washed twice with 1 ml PBS and irradiated, with a dose of 960 J/m^2 UVB, as described in section 2.3.20. After 48 hr the cells were harvested for trypan blue assay as described in section 2.3.14.

4.2.17 The ability of gold thioglucose supplementation to modify the susceptibility of HaCaT cells to cytotoxicity resulting from UVB exposure

HaCaT cells were passaged into 24 well plates using Se-deficient medium. The cells were then left to grow for 48 hr. Se-deficient medium to which GTG concentrations (1, 10, or 100 μM) had been added was then placed on the cells. After an incubation period of 48 hr, the cells were washed twice with 1 ml PBS and irradiated, with a dose of 720 J/m^2 UVB, as described in section 2.3.20. After 48 hr the cells were harvested for trypan blue assay as described in section 2.3.14.

4.2.18 Investigation of potential TR inhibitors using the DTNB assay system

Stock solutions of azelaic acid and N-ethylmalemide (NEM) were prepared in ethanol, and those of anthralin and 13-cis retinoic acid (13-cis RA) were prepared in acetone. The final concentrations of inhibitors in human placental cytosol were: azelaic acid 0.01, 0.02, 0.06, 0.11, 0.22, 0.56, 1.11 mM; anthralin 0.01, 0.03, 0.06, 0.11, 0.28 mM; NEM 0.01, 0.02, 0.03, 0.04, 0.06, 0.11 mM; 13-cis RA 0.01, 0.03, 0.06, 0.11, 0.28, 0.56 mM. After an overnight incubation at 4°C, TR activity was measured using the DTNB assay as detailed in section 2.3.5.1. Blanks for ethanol and acetone were run, along with blanks for the appropriate inhibitor compounds.

4.2.19 Comparison of potential TR inhibitors in the DTNB assay system and the insulin assay system for TR

Fresh stock solutions of inhibitor compounds were prepared as described in section 4.2.18 above, and added to human placental cytosol to give the same final concentrations as detailed in section 4.2.18. In addition, p-chloromercuri-benzoic acid (PCMB) was added at concentrations of 0.01, 0.03, 0.06, 0.11, 0.28 mM to human placental cytosol. After an overnight incubation at 4°C, TR activity was measured in the cytosols using both the DTNB assay system (section 2.3.5.1) and the insulin reduction assay system (section 2.3.5.2).

4.2.20 Statistical analysis

One-way analysis of variance (ANOVA) was used to test for significant differences in LDH retention in response to different concentrations of menadione, and in % trypan blue stained cells in response to UVB irradiation. In the event that the variation was significant ($p < 0.05$), a Tukey-Kramer multiple comparisons post-test was used to test for the level of significance of differences in % LDH retained. The different groups of cells (e.g. Se-deficient cells versus Se-supplemented cells) were compared at individual menadione concentrations using the Student's t-test for unpaired data. One-way ANOVA and a Tukey-Kramer multiple comparisons post-test were also used to investigate significant differences between levels of selenoprotein expression and activity in cells cultured in different concentrations of sodium selenite or selenomethionine. In the event of large SDs in a particular data set (> 3 SDs from the mean), the data was log transformed prior to ANOVA evaluation. The expression of selenoenzymes in cells at different confluence levels was compared using the Student's t-test for unpaired data.

4.3 RESULTS

All graphs presented are the data from a single experiment, using culture flasks/wells in triplicate, unless stated otherwise.

4.3.1 The effect of sodium selenite or selenomethionine supplementation on intracellular selenoprotein expression and activity in HaCaT cells

a) Sodium selenite supplementation

In the first experiment, incubation with 10 nM sodium selenite resulted in a significant induction of TR (Figure 4.01 a and b), and cyGPX (Figure 4.01 d) ($p < 0.05$). Although PHGPX activity appeared to be induced by concentrations of 50 nM and 1000 nM sodium selenite, (Figure 4.01 c) the differences did not achieve statistical significance due to large standard deviations on the data. The maximal increase in TR activity was a 4.0-fold (n=3) increase measured in HaCaT cells supplemented with 10 nM sodium selenite compared to that measured in Se-deficient cells. Increasing the selenite concentration above 10 nM had no further significant effect on induction of TR activity above that seen with 10 nM selenite. Figure 4.01 d shows that significant induction of cyGPX activity was first achieved by supplementation of HaCaT cells with 10 nM sodium selenite ($p < 0.05$), which increased the activity by 2.3-fold over that seen in Se-deficient control cells. CyGPX activity was maximally induced by 100 nM sodium selenite, resulting in 4.8-fold ($p < 0.001$) the activity of that seen in control cells. A sodium selenite concentration of 200 nM to 1000 nM had no further effect on cyGPX activity. LDH release did not indicate cytotoxicity at any of the selenite concentrations used.

In the second experiment, TR activity was again optimally induced by 10 nM sodium selenite ($p < 0.001$) (figure 4.02 b). PHGPX activity increased to 1.6-fold that of Se-deficient control cells when HaCaT cells were supplemented with 10 nM sodium selenite, but again this failed to reach statistical significance due to wide variation in the response in individual flasks. Similarly, the cyGPX activity differences from control activity were not statistically significant at any sodium selenite concentration tested due to high standard deviation found in this experiment. However, the trend towards decreasing cyGPX activity at concentrations higher than 100 nM sodium selenite was again observed. The cyGPX activity of cells supplemented with 1000 nM sodium selenite was not statistically significantly different to that of the Se-deficient control cells.

b) Selenomethionine supplementation

Concentrations of 10, 100, 200 and 1000 nM selenomethionine increased TR, PHGPX and cyGPX activities in a concentration-dependent manner (figure 4.03). The increase in activity in TR and PHGPX activities above basal levels was statistically significant only at 1000 nM selenomethionine ($p < 0.05$). CyGPX activity was significantly increased over basal levels by 200 nM and 1000 nM selenomethionine ($p < 0.01$). None of the selenoenzyme activities appeared to have reached a plateau by 1000 nM selenomethionine.

HaCaT cells supplemented with selenomethionine concentrations extending to 100,000 nM showed a different pattern of selenoprotein expression (figure 4.04). TR activity was increased over that seen in control cells by all concentrations of selenomethionine tested, and showed maximal expression at 10,000 nM selenomethionine ($p < 0.05$). PHGPX activity showed maximal expression at 1000 nM selenomethionine, although this failed to reach statistical significance. At selenomethionine concentrations of 10,000 and 100,000 nM, the PHGPX activity fell to below the level of activity seen at 1000 nM selenomethionine, and was not significantly different to control levels with 100,000 nM. CyGPX activity increased in a concentration-dependent manner, reaching maximal expression at 1000 nM selenomethionine ($p < 0.001$). However, the level of activity decreased slightly with 10,000 nM selenomethionine. Using 100,000 nM selenomethionine, there was no significant difference compared to the basal level in control cells. LDH release did not indicate any cytotoxicity at any concentration tested of selenomethionine to HaCaT cells.

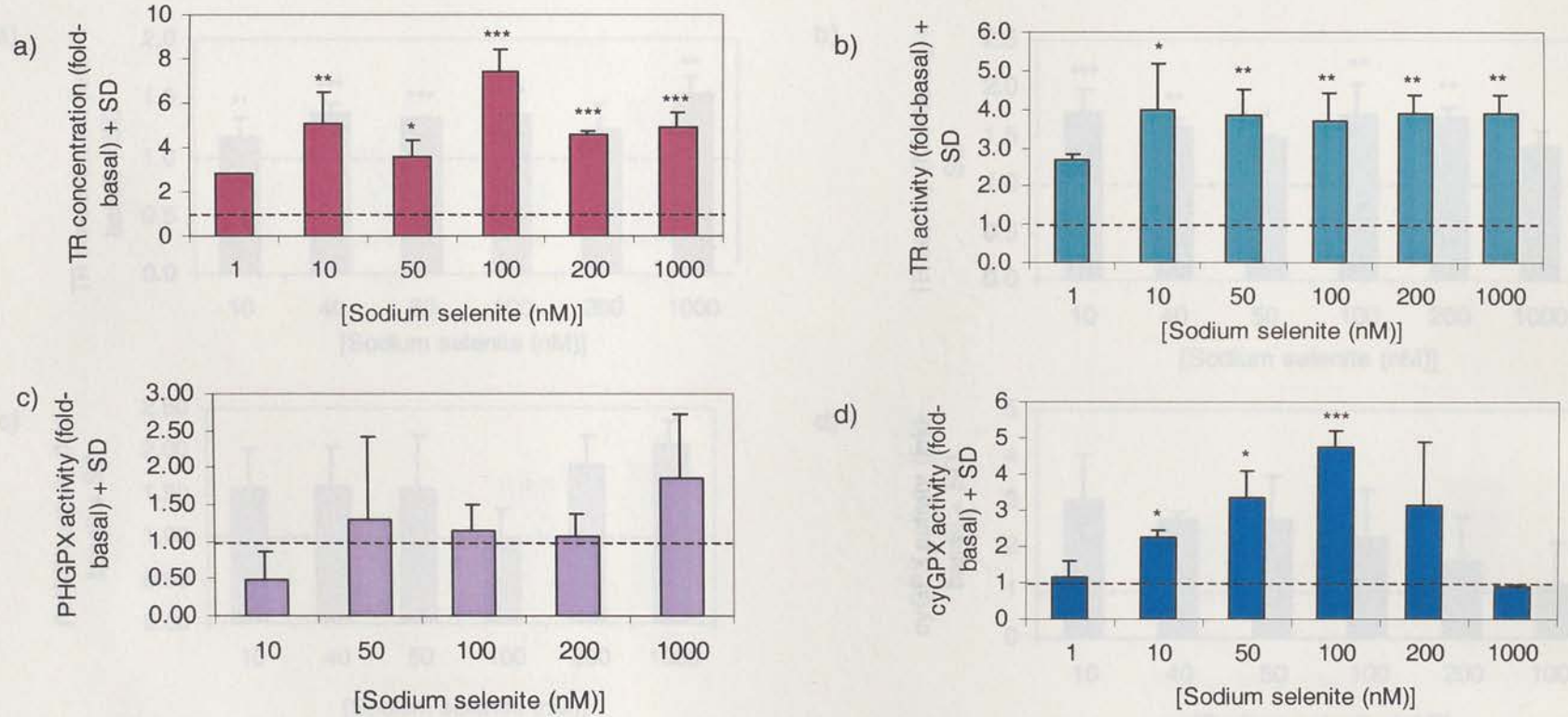


Figure 4.01 Thioredoxin reductase (TR) mass (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in HaCaT cells supplemented with sodium selenite for 48 hr. Results shown are those of the means of three flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. control cells. The 1 nM sodium selenite data is missing from the PHGPX activity data due to the loss of the sample.

4-17

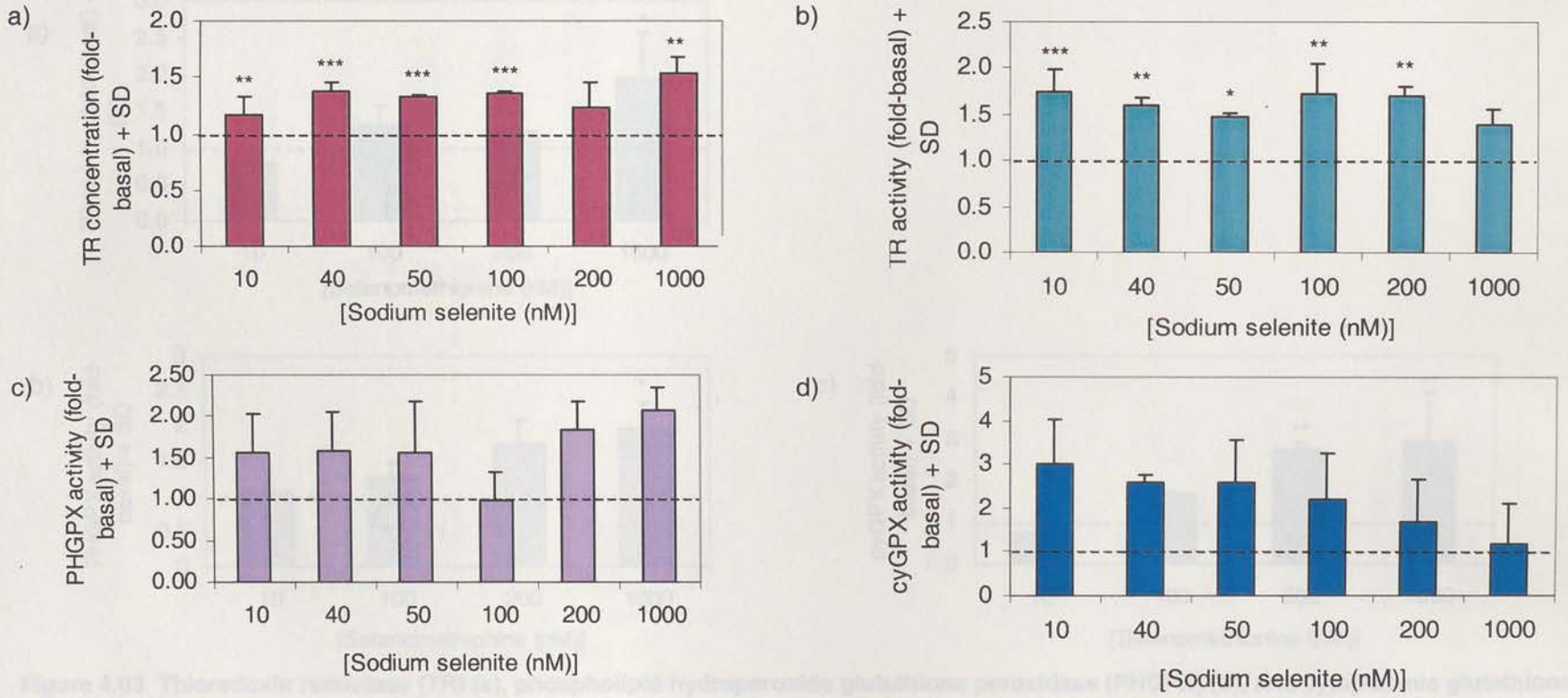


Figure 4.02 Thioredoxin reductase (TR) mass (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in HaCaT cells supplemented with sodium selenite for 48 hr. Results shown are those of the means of three flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. control cells.

4-18

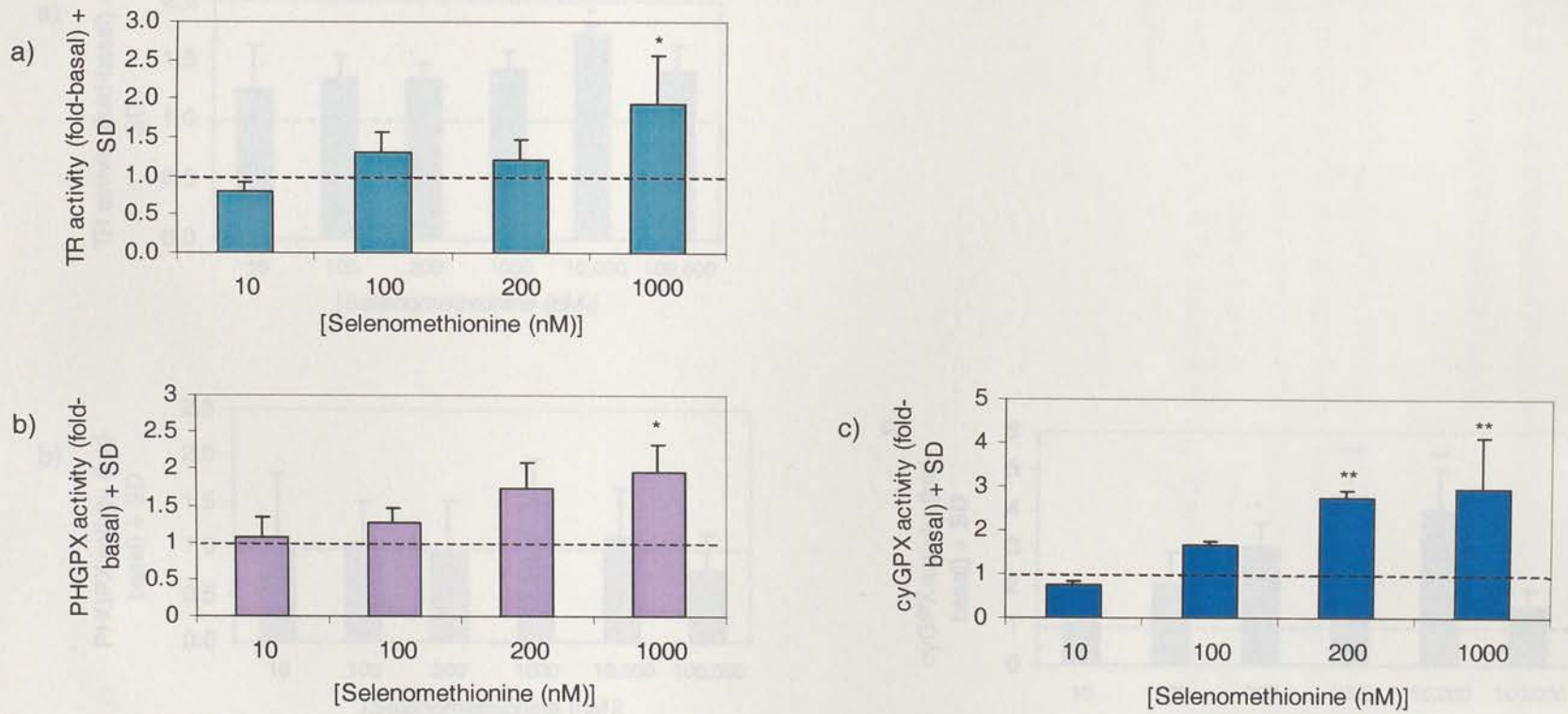


Figure 4.03 Thioredoxin reductase (TR) (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in HaCaT cells supplemented with selenomethionine (0, 10, 100, 200, 1000 nM) for 48 hr. Results shown are those of the means of three flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**.

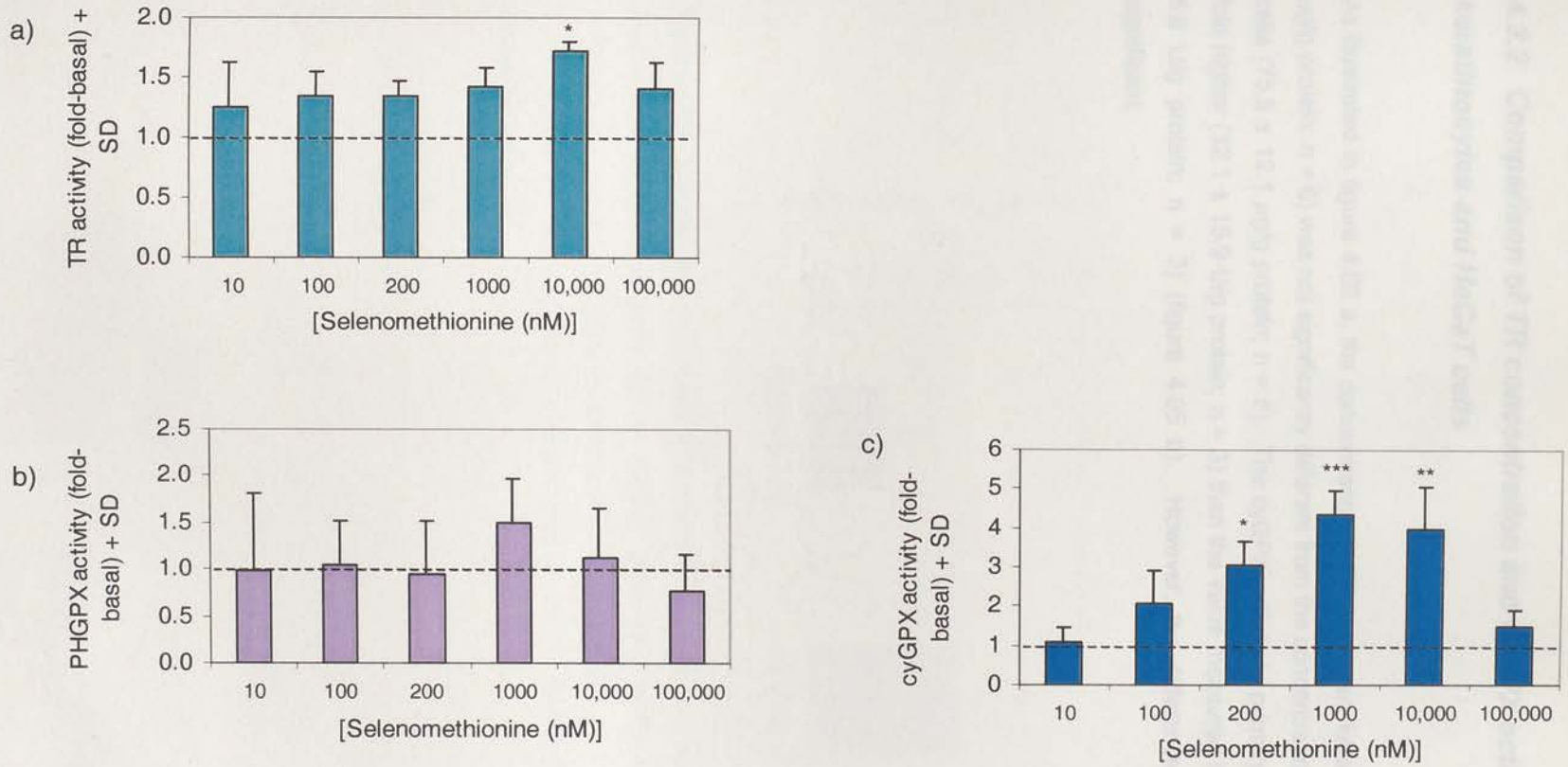


Figure 4.04 Thioredoxin reductase (TR) (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in HaCaT cells supplemented with selenomethionine (0, 10, 100, 200, 1000, 10,000, 100,000 nM) for 48 hr. Results shown are those of the means of three flasks + SD. The respective basal level of each selenoenzyme is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. control cells.

4.3.2 Comparison of TR concentration and cyGPX activity in primary keratinocytes and HaCaT cells

As illustrated in figure 4.05 a, the concentration of TR in primary keratinocytes (81.5 ± 14.4 μg/g protein; n = 6) was not significantly different from the concentration measured in HaCaT cells (75.8 ± 12.1 μg/g protein; n = 6). The cyGPX activity in primary keratinocytes was 2-fold higher (32.1 ± 15.9 U/g protein; n = 3) than the value measured in HaCaT cells (16.6 ± 6.8 U/g protein; n = 3) (figure 4.05 b). However, the difference was not statistically significant.

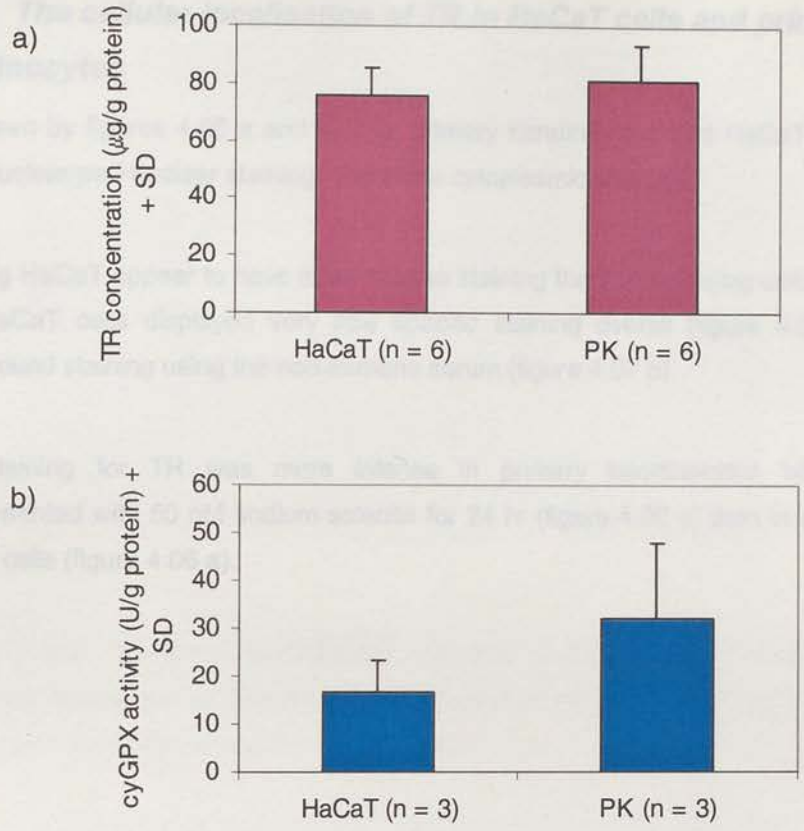


Figure 4.05 Thioredoxin reductase (TR) concentration (a) and cytoplasmic glutathione peroxidase (cyGPX) activity (b) in HaCaT cells and primary keratinocytes. TR concentration was measured by radioimmunoassay in cells grown in Se-deficient medium in 6 well plates for the radioimmunoassay, and in 75cm^2 flasks for the cyGPX activity measurements.

4.3.3 The cellular localisation of TR in HaCaT cells and primary keratinocytes

As shown by figures 4.06 a and 4.07 a, primary keratinocytes and HaCaT cells appear to have nuclear/peri-nuclear staining, and some cytoplasmic staining.

Dividing HaCaT appear to have more intense staining than non-dividing cells (figure 4.07 a). The HaCaT cells displayed very little specific staining overall (figure 4.07 a), with high background staining using the non-immune serum (figure 4.07 b).

The staining for TR was more intense in primary keratinocytes which had been supplemented with 50 nM sodium selenite for 24 hr (figure 4.06 c) than in unsupplemented control cells (figure 4.06 a).

Figure 4.06a Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of human primary keratinocytes. x 100 magnification. Both the primary and secondary antibodies were present.

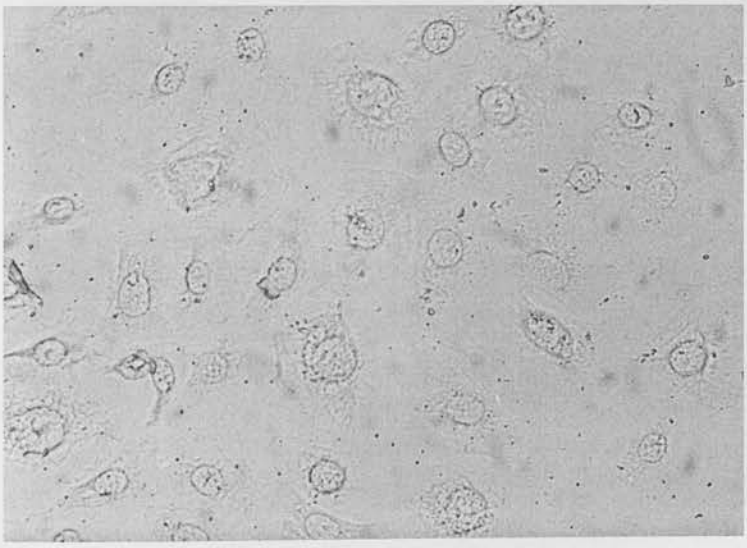


Figure 4.06b Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of human primary keratinocytes. x 100 magnification. For the immunohistochemistry here the secondary antibody was present, but without the primary antibody.

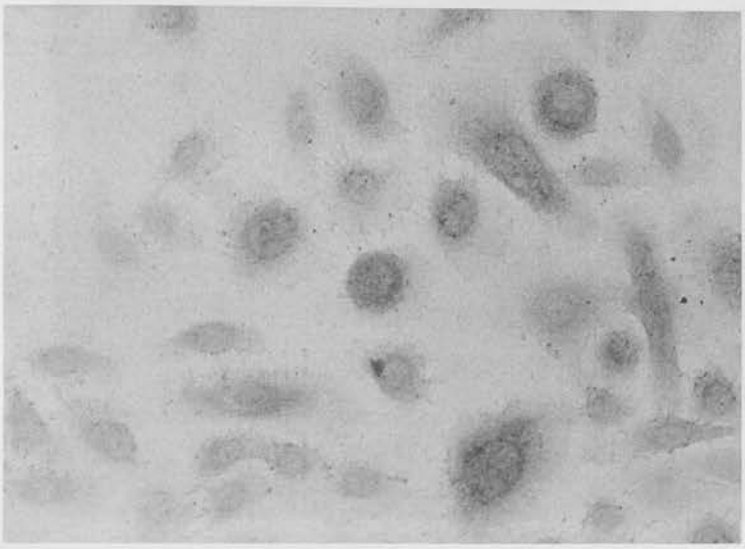


Figure 4.06 c Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of human primary keratinocytes. x 100 magnification. The primary keratinocytes were supplemented with 50 nM Se for 24 hr prior to immunohistochemistry.

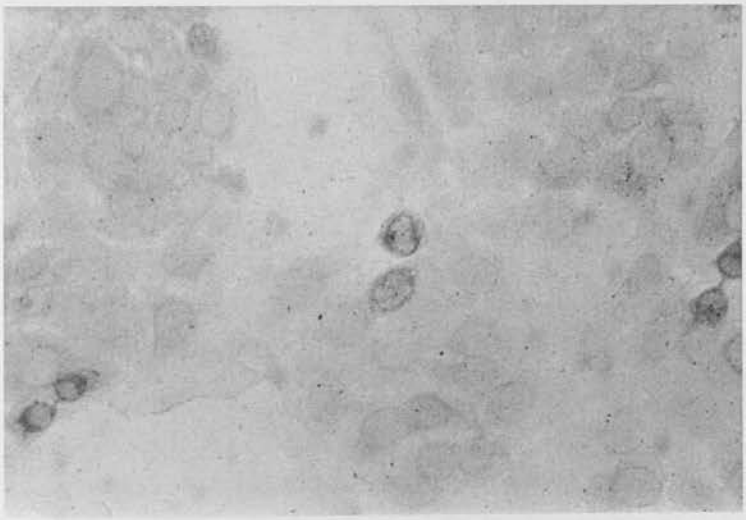


Figure 4.07a Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of HaCaT cells. x 100 magnification. Both the primary and secondary antibodies were present.

Figure 4.07b Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of HaCaT cells. x 100 magnification. Non-reactive serum was applied as a control.

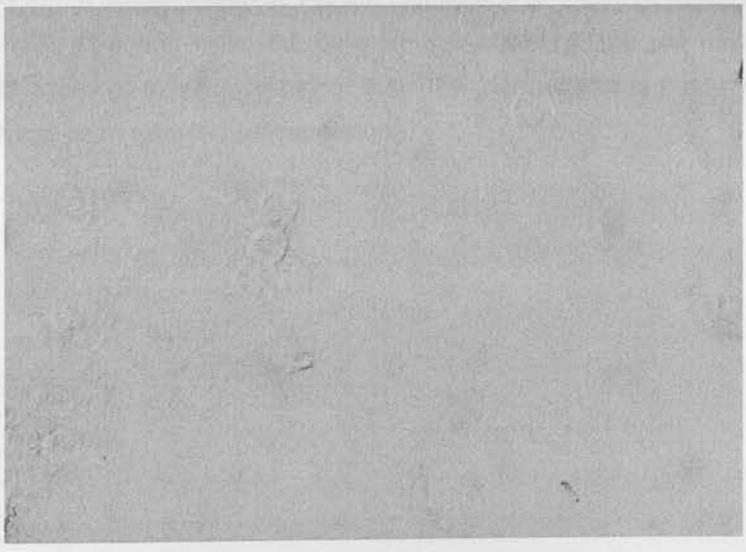


Figure 4.07c Immunohistochemical staining of thioredoxin reductase in a non-confluent monolayer of HaCaT cells. x 100 magnification. For the immunohistochemistry here the secondary antibody was present, but without the primary antibody.

4.3.4 The effect of different concentrations of menadione on LDH retention in HaCaT cells cultured in selenium-deficient medium

The viability of HaCaT cells was decreased in a dose-dependent manner in response to treatment with menadione (figure 4.08 a and b). In the first experiment (figure 4.08 a), the cytotoxic effect of menadione was first evident at a concentration of 100 μM, resulting in 66.83 ± 5.84 % LDH retention (mean ± SD, n =3) ($p < 0.01$ cf. control cells). A further decrease in cell viability was measured when cells were exposed to 150 μM menadione, resulting in 9.50 ± 8.02 % LDH retention ($p < 0.001$ cf. control cells). Concentrations exceeding 150 μM menadione (200, 250, 300 μM) gave 100 % cell damage such that no LDH retention could be measured in the cells. A second cytotoxicity dose-response curve was then performed to expand the concentration range.

In the second dose-response experiment (figure 4.08 b), cytotoxicity was initially observed at 80μM menadione (83.79 ± 3.92 % LDH retention; mean ± SD, n=3) ($p < 0.01$ cf. control cells). A concentration of 100 μM menadione further decreased cell viability to 40.23 ± 5.62 % LDH retention ($p < 0.001$ cf. control cells). Cell viability was measured to be 11.47 ± 2.61 % LDH retention with a concentration of 140 μM menadione ($p < 0.001$), which decreased to 6.22 ± 1.77 % LDH retention when the cells were exposed to 160 μM menadione ($p < 0.001$). Concentrations of menadione higher than 160 μM menadione produced no further cytotoxicity than that seen with 160 μM menadione.

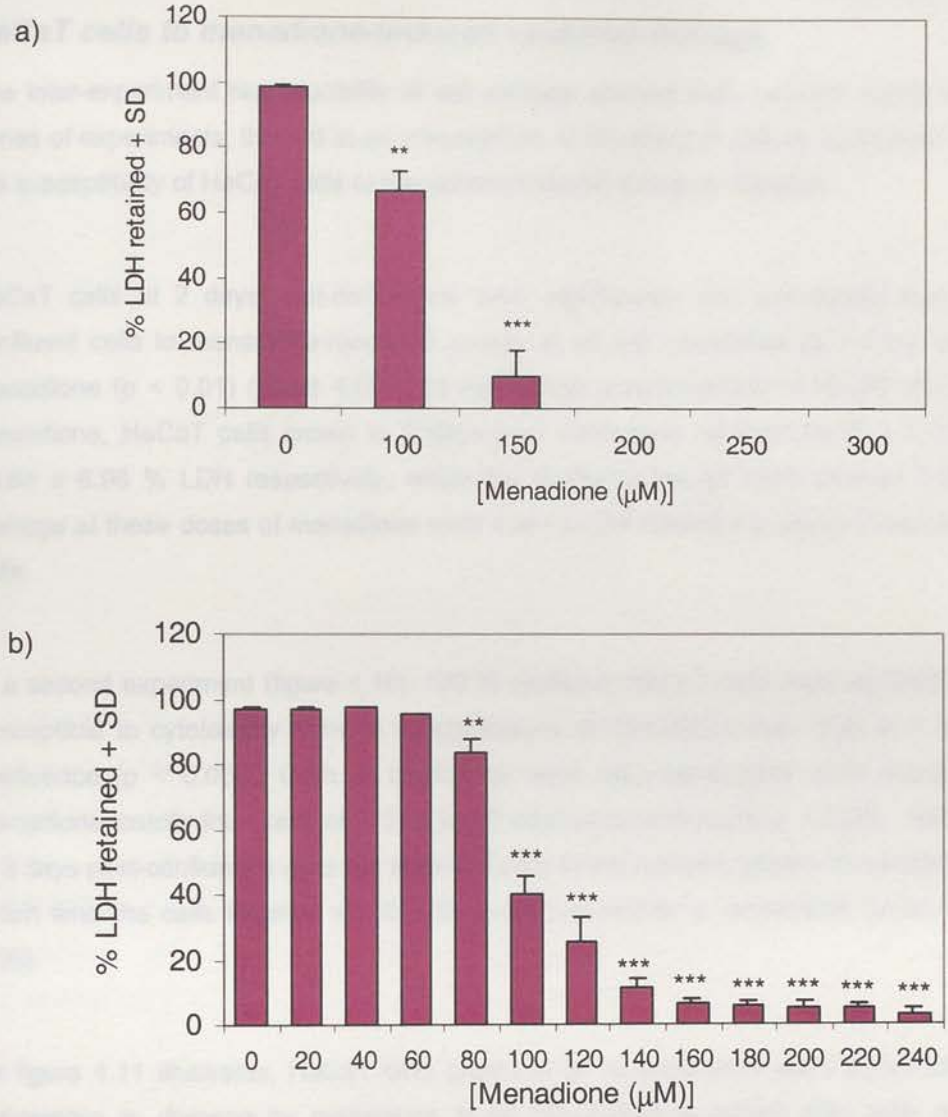


Figure 4.08 The effect of menadione on % LDH retained in HaCaT cells after 18 hr exposure. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of menadione (a) (0, 100, 150, 200, 250, 300 μM) (b) (0 to 240 μM, in 20 μM increments) when confluent. Results shown are those of the mean of triplicate wells + SD. $p < 0.01$** ; $p < 0.001$*** cf. control cells receiving 0 μM menadione.

4.3.5 The effect of cellular confluence level on the susceptibility of HaCaT cells to menadione-induced oxidative damage

The inter-experiment reproducibility of cell damage showed high variation during the initial series of experiments; this led to an investigation of the effect of cellular confluence level on the susceptibility of HaCaT cells to menadione-induced oxidative damage.

HaCaT cells at 2 days post-confluence were significantly less susceptible than 100 % confluent cells to menadione-mediated toxicity at 40 μM menadione ($p < 0.05$) or 60 μM menadione ($p < 0.01$) (figure 4.09). At menadione concentrations of 80 μM and 100 μM menadione, HaCaT cells grown to 2 days post confluence retained 68.37 ± 2.12 % and 36.64 ± 6.98 % LDH respectively, whilst the confluent HaCaT cells showed 100 % cell damage at these doses of menadione such that no LDH retention could be measured in the cells.

In a second experiment (figure 4.10), 100 % confluent HaCaT cells were significantly more susceptible to cytotoxicity from all concentrations of menadione than cells at 1 day post-confluence ($p < 0.05$). Cells at confluence were also significantly more susceptible to menadione toxicity than cells at 2, 3, 4 and 5 days post-confluence ($p < 0.05$). HaCaT cells at 3 days post-confluence were the most resistant to the cytotoxic effects of menadione, after which time the cells became significantly more susceptible to menadione cytotoxicity ($p < 0.05$).

As figure 4.11 illustrates, HaCaT cells grown to 50 % confluence were significantly more susceptible to damage by menadione at all concentrations tested than cells at 100 % confluence, 1, 2, or 4 days post-confluence ($p < 0.01$). Cells at 100 % confluence suffered significant cytotoxicity at a concentration of 40 μM menadione ($p < 0.001$), but cells at 1, 2, or 4 days post-confluence did not suffer any significant damage. HaCaT cells at 2 days post-confluence tended to be the most resistant to the cytotoxic effects of menadione.

A cell density plating protocol was employed following these investigations to decrease the variation in the response of cells due to confluence differences. The HaCaT cells were passaged at a cell density of 2×10^5 cells/cm^2, which took 4 days to reach confluence.

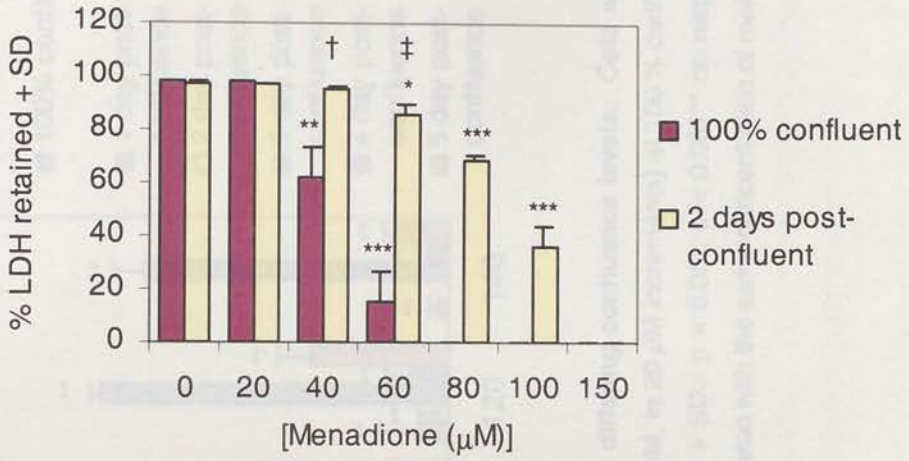


Figure 4.09 The effect of menadione on % LDH retained in HaCaT cells grown to differing confluence levels. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of menadione (0, 20, 40, 60, 80, 100, 150 μM) at 100% confluence or at 2 days post-confluence. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$** ; $p < 0.001$*** cf. respective control cells receiving 0 μM menadione. $p < 0.05$†; $p < 0.01$‡ cf. 100 % confluent cells at the same menadione concentration.

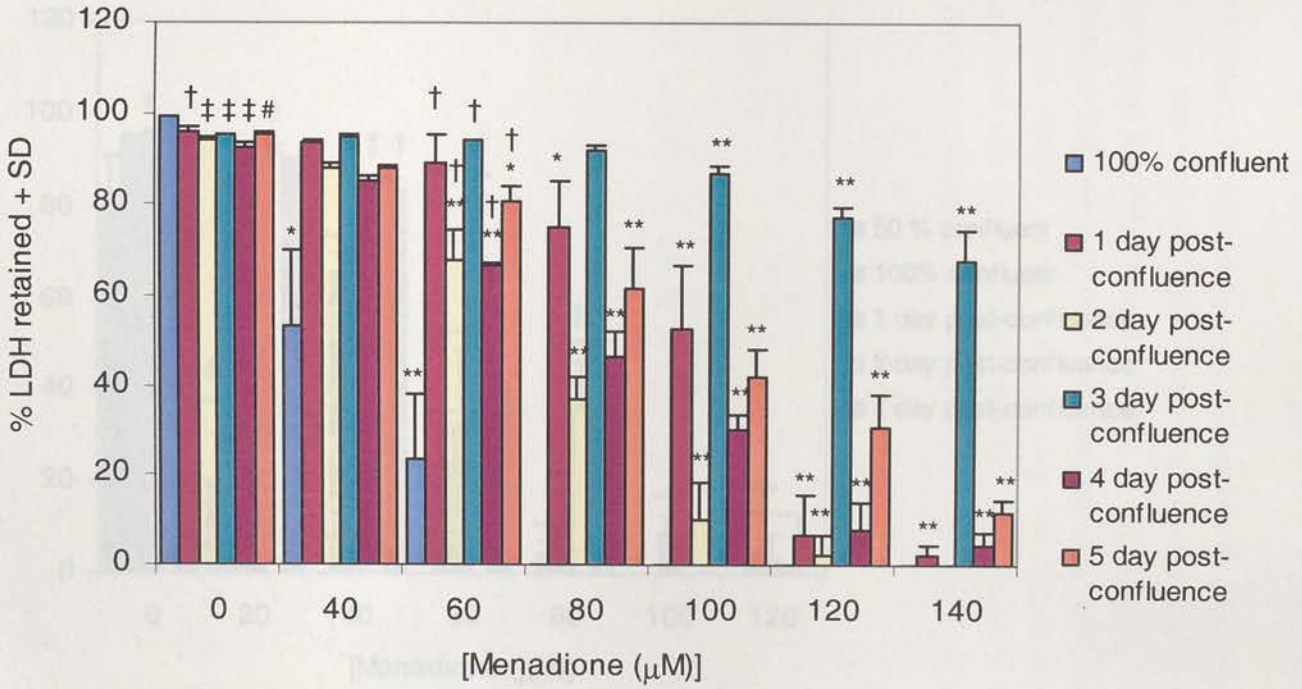


Figure 4.10 The effect of menadione on % LDH retained in HaCaT cells grown to differing confluence levels. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of menadione (0 to 140 μM, in 20 μM increments) at 100 % confluence or at 1, 2, 3, 4, or 5 days post-confluence. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.001$** cf. respective control cells receiving 0 μM menadione. $p < 0.05$†, $p < 0.01$‡, $p < 0.001$# cf. 100 % confluent cells treated with the same concentration of menadione.

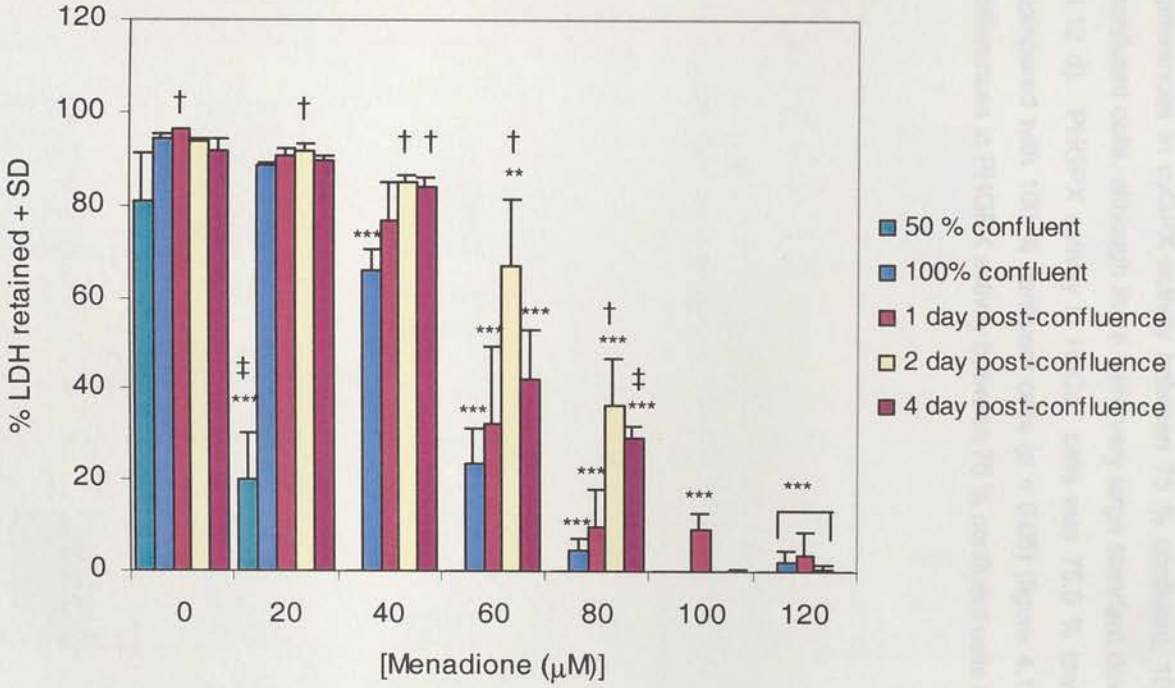


Figure 4.11 The effect of menadione on % LDH retained in HaCaT cells grown to differing confluence levels. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of menadione (0 to 140 μM, in 20 μM increments) at 50% or 100% confluence or at 1, 2, or 4 days post-confluence. Results shown are those of the mean of triplicate wells + SD. $p < 0.01$** ; $p < 0.001$*** cf. respective control cells receiving 0 μM menadione. $p < 0.05$†, $p < 0.01$‡ cf. 100 % confluent cells treated with the same menadione concentration.

4.3.6 The effect of cellular confluence level on TR expression and activity, and cyGPX and PHGPX activity of HaCaT cells

TR concentration and activity did not significantly differ between 75 % confluent, 100 % and 2 day post-confluent HaCaT cells (figure 4.12 a and b). There were no significant differences in cyGPX activity between 75 % confluent, 100 % confluent and 2 day post-confluent cells, although there were very large standard deviations in this data group (figure 4.12 d). PHGPX activity in HaCaT cells was 75.9 % lower in 2 day-post confluent cells compared with 100 % confluent cells ($p < 0.05$) (figure 4.12 c). There were no significant differences in PHGPX activity between 75 % confluent cells and 100 % confluent cells.

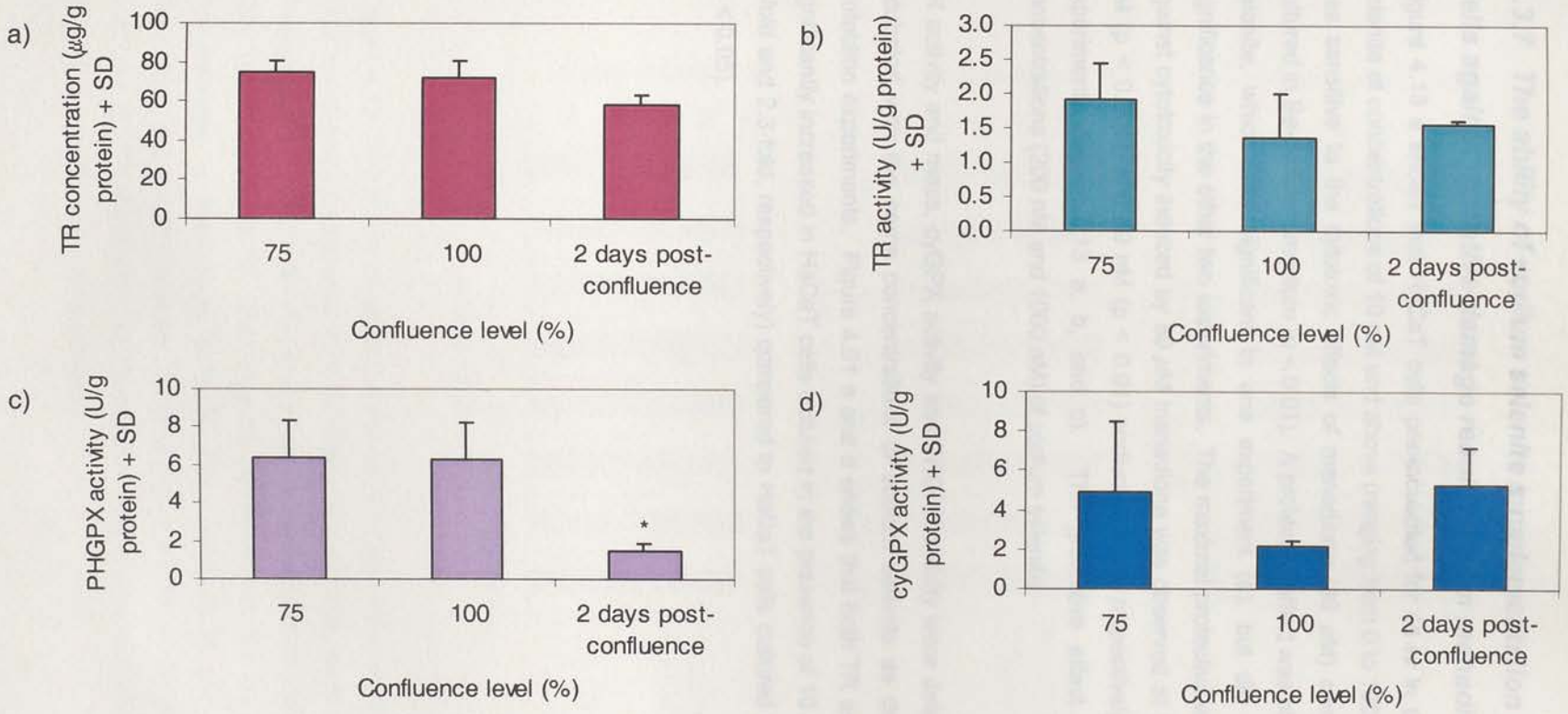


Figure 4.12 TR mass (a) and activity (b), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (c), and cytoplasmic glutathione peroxidase (cyGPX) activity (d) in HaCaT cells grown to differing confluence levels. Results shown are those of the mean of three flasks + SD. $p < 0.05$* cf. 100% confluent cells.

4.3.7 The ability of sodium selenite supplementation to protect HaCaT cells against oxidative damage resulting from menadione exposure

Figure 4.13 a shows that HaCaT cells pre-incubated for 48 hr in the presence of sodium selenite at concentrations of 10 nM and above (ranging from 0 to 1000 nM) were significantly less sensitive to the cytotoxic effects of menadione (80 μM) compared to HaCaT cells cultured in Se-deficient medium ($p < 0.01$). A protective effect was observed at 1 nM sodium selenite, which was significant in one experiment (a), but did not achieve statistical significance in the other two experiments. The maximal protective effect of sodium selenite against cytotoxicity induced by 80 μM menadione was observed at 10 nM ($p < 0.01$), 1000 nM ($p < 0.001$), and 40 nM ($p < 0.01$) sodium selenite respectively for the three different experiments (figure 4.13 a, b, and c). The protective effect was not lost at high concentrations (200 nM and 1000 nM) of sodium selenite.

TR activity and mass, cyGPX activity and PHGPX activity were determined in HaCaT cells incubated with the same concentrations of sodium selenite as described above for the protection experiments. Figure 4.01 a and d shows that both TR and cyGPX activity were significantly increased in HaCaT cells cultured in the presence of 10 nM sodium selenite (by 4-fold and 2.3-fold, respectively) compared to HaCaT cells cultured in Se-deficient medium ($p < 0.05$).

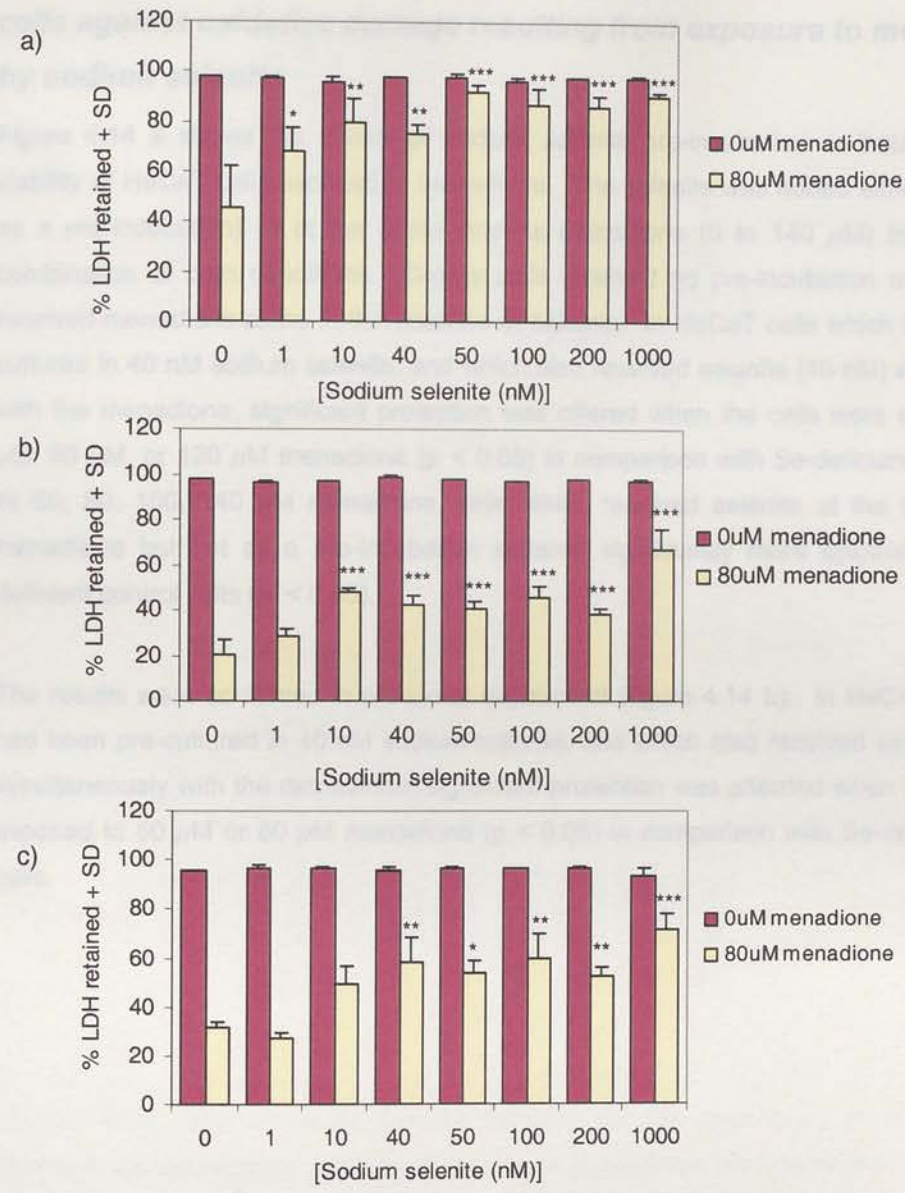


Figure 4.13 The effect of sodium selenite pre-incubation on the susceptibility of HaCaT cells to menadione-induced cell damage. HaCaT cells were incubated with Se-deficient medium supplemented with different concentrations of sodium selenite (0, 1, 10, 40, 50, 100, 200, 1000 nM) for 48 hr. Following the pre-incubation the HaCaT cells were exposed to concentrations of menadione (0 or 80 μM). After 18 hr cell viability was assessed by determining the % LDH retained. Results shown are the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. Se-deficient control cells receiving 0 μM menadione. Graphs (a) – (c) are each a separate experiment.

4.3.8 The requirement for pre-incubation in the protection of HaCaT cells against oxidative damage resulting from exposure to menadione by sodium selenite

Figure 4.14 a shows the effects of sodium selenite pre-incubation or incubation on the viability of HaCaT cells exposed to menadione. The selenite was added either prior to (i.e. as a pre-incubation) or at the same time as menadione (0 to 140 μM) treatment, or a combination of both conditions. Control cells received no pre-incubation of selenite and received menadione alone in the absence of selenite. In HaCaT cells which had been pre-cultured in 40 nM sodium selenite, and which also received selenite (40 nM) simultaneously with the menadione, significant protection was offered when the cells were exposed to 40 μM, 80 μM, or 120 μM menadione ($p < 0.05$) in comparison with Se-deficient control cells. At 60, 80, 100, 140 μM menadione, cells which received selenite at the same time as menadione but not as a pre-incubation suffered significantly more cytotoxicity than Se-deficient control cells ($p < 0.05$).

The results were confirmed in a second experiment (figure 4.14 b). In HaCaT cells which had been pre-cultured in 40 nM sodium selenite, and which also received selenite (40 nM) simultaneously with the menadione, significant protection was afforded when the cells were exposed to 60 μM or 80 μM menadione ($p < 0.05$) in comparison with Se-deficient control cells.

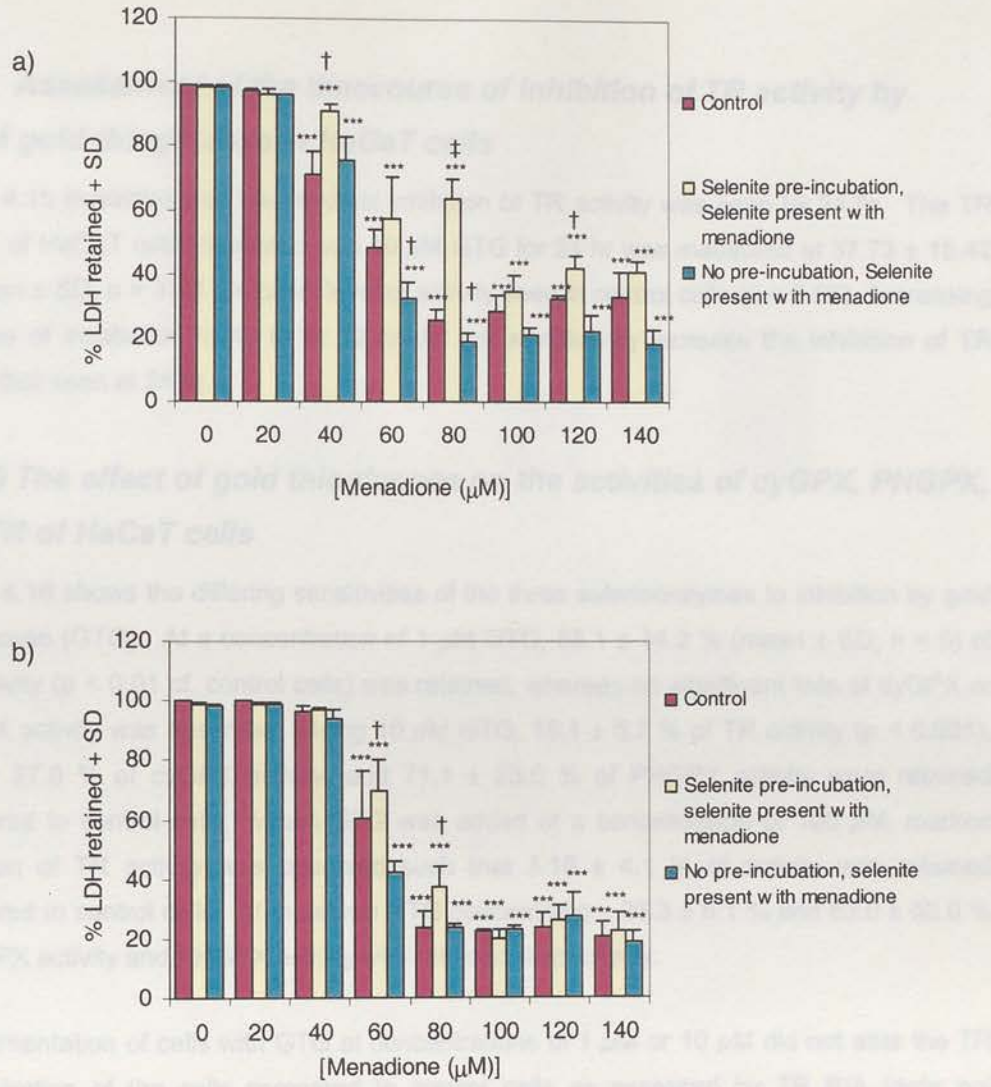


Figure 4.14 The effect of sodium selenite on the sensitivity of HaCaT cells to menadione cytotoxicity added either prior to menadione treatment or at the same time as menadione treatment. HaCaT cells were pre-incubated with either Se-deficient medium supplemented with sodium selenite (40 nM) or Se-deficient medium with no addition. After 48 hr incubation the medium was removed and replaced with medium containing 40 nM sodium selenite and menadione (0, 20, 40, 60, 80, 100, 120, 140 μM) or menadione unsupplemented with selenite for 20 hr. Control cells received no menadione or Se supplementation. Results shown are the mean of triplicate wells + SD. $p < 0.001$*** cf. control cells receiving 0 μM menadione. $p < 0.05$†; $p < 0.001$‡ cf. control (Se-deficient) cells at the same menadione concentration. Graphs (a) and (b) are each a separate experiment.

4.3.9 Assessment of the timecourse of inhibition of TR activity by 10 μM gold thioglucose in HaCaT cells

Figure 4.15 illustrates that the maximal inhibition of TR activity was seen by 24 hr. The TR activity of HaCaT cells incubated with 10 μM GTG for 24 hr was measured at 37.73 ± 15.42 % (mean ± SD, n = 3) of the basal level of activity seen in control cells ($p < 0.05$). Increasing the time of incubation to 48 hr or 72 hr did not significantly increase the inhibition of TR above that seen at 24 hr.

4.3.10 The effect of gold thioglucose on the activities of cyGPX, PHGPX, and TR of HaCaT cells

Figure 4.16 shows the differing sensitivities of the three selenoenzymes to inhibition by gold thioglucose (GTG). At a concentration of 1 μM GTG, 85.1 ± 14.2 % (mean ± SD, n = 6) of TR activity ($p < 0.01$ cf. control cells) was retained, whereas no significant loss of cyGPX or PHGPX activity was observed. Using 10 μM GTG, 18.1 ± 5.7 % of TR activity ($p < 0.001$), 93.0 ± 27.0 % of cyGPX activity, and 71.1 ± 23.0 % of PHGPX activity were retained compared to control cells. When GTG was added at a concentration of 100 μM, marked inhibition of TR activity was observed such that 3.18 ± 4.1 % of activity was retained compared to control cells. At the same GTG concentration, 33.3 ± 6.1 % and 63.0 ± 53.0 % of cyGPX activity and PHGPX activity was retained respectively.

Supplementation of cells with GTG at concentrations of 1 μM or 10 μM did not alter the TR concentration of the cells compared to control cells as assessed by TR RIA (data not shown). There are no methods currently available to us to assess cyGPX concentration.

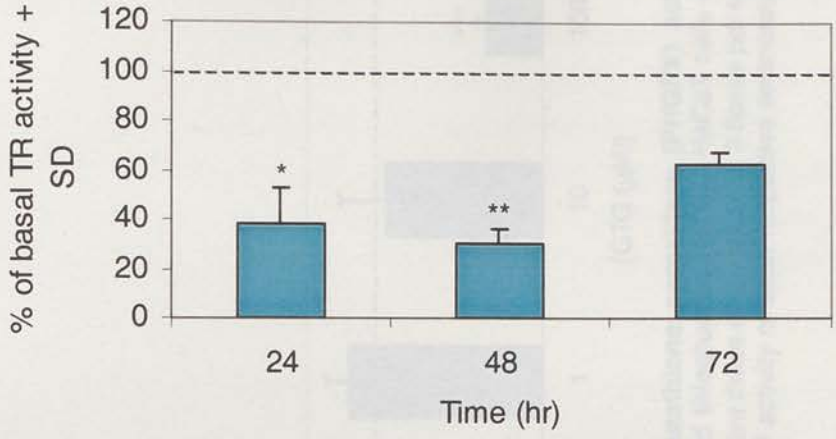


Figure 4.15 Timecourse of inhibition of thioredoxin reductase (TR) activity by gold thioglucose (GTG) in HaCaT cells. Cells were incubated with Se-deficient medium supplemented with GTG (10 μM) for 24, 48, and 72 hr respectively. Results shown are those of the mean of 3 flasks + SD. The basal level of activity in control cells is indicated by the dashed line. $p < 0.05$ *; $p < 0.01$ ** cf. control cells.

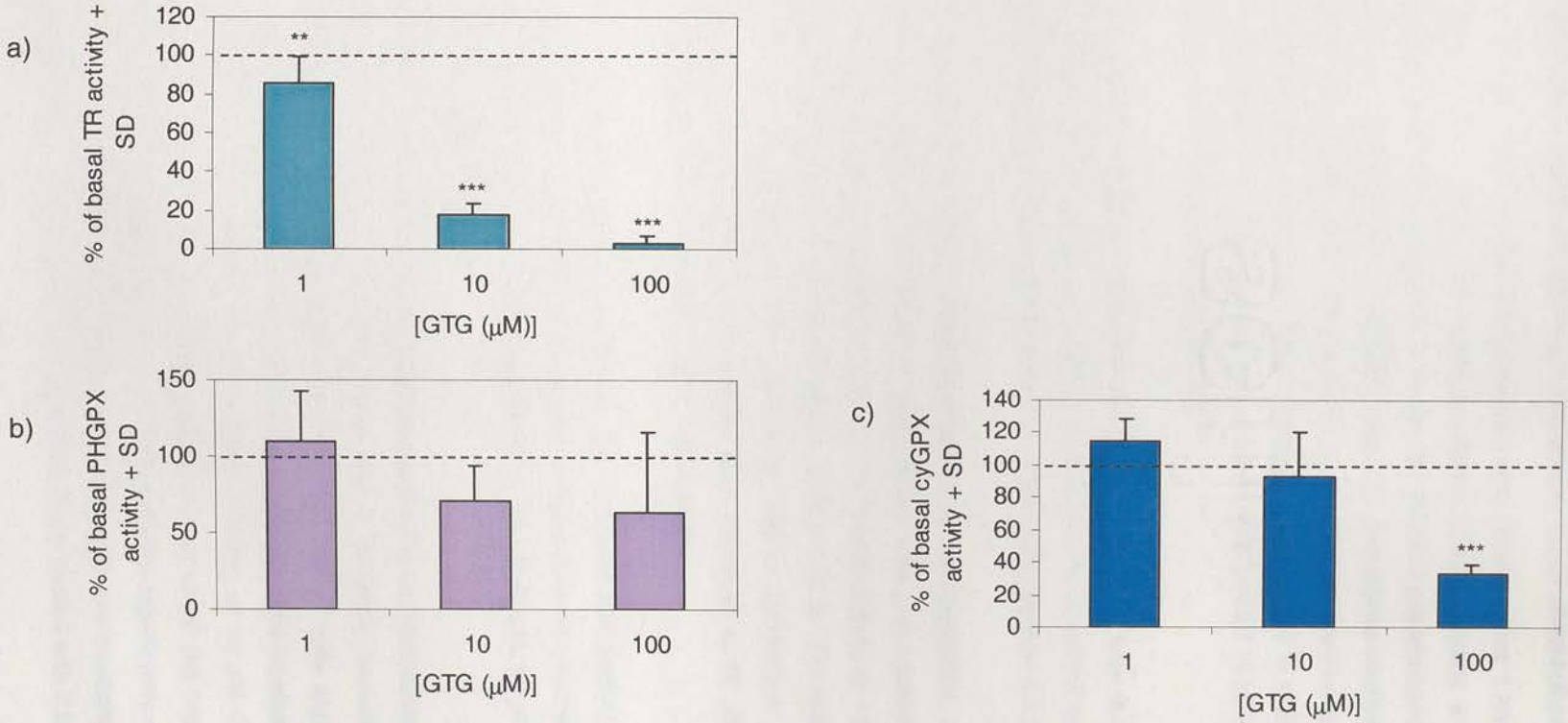


Figure 4.16 Thioredoxin reductase (TR) activity (a), phospholipid hydroperoxide glutathione peroxidase (PHGPX) activity (b), and cytoplasmic glutathione peroxidase (cyGPX) activity (c) in HaCaT cells treated with gold thioglucose (GTG). HaCaT cells were incubated with Se-deficient medium supplemented with GTG (0, 1, 10, 100 μM) for 48 hr. Results shown are those of the mean of 3 flasks per experiment, of 2 separate experiments meaned, assayed in the same run on the same day. The basal level of activity of each respective selenoenzyme in control cells is indicated by the dashed line. $p < 0.01$ **; $p < 0.001$ *** cf. control cells.

4.3.11 The effect of gold thioglucose on the susceptibility of HaCaT cells to oxidative damage resulting from menadione exposure

A concentration of 100 μM menadione was chosen since it had previously been shown to result in 50 – 60 % LDH retention (figure 4.08), providing an opportunity to see altered viability with GTG. Figure 4.17 shows the effect of pre-incubation with 10 μM GTG for 48 hr on the susceptibility of HaCaT cells to menadione-mediated damage. At 100 μM menadione, cells which had received a 10 μM GTG pre-incubation were significantly more susceptible ($p < 0.05$) to cytotoxic damage (3.74 ± 3.36 % LDH retained) than control cells which had received no GTG pre-incubation (19.83 ± 6.27 % LDH retained) but were exposed to the same menadione concentration.

Incubation of HaCaT cells with 10 μM GTG for 48 hr was found to result in 18.1 ± 5.7% retention of TR activity ($p < 0.001$), 93.0 ± 27.0 % of cyGPX activity, and 71.1 ± 23.0 % of PHGPX activity retention compared to control cells (section 4.3.10, above; figure 4.16).

In a second experiment, when exposed to 40 μM menadione, control HaCaT cells retained 66.91 ± 3.53 % LDH activity; in comparison, cells pre-incubated with 10 μM GTG prior to menadione exposure retained 24.05 ± 2.84 % LDH activity ($p < 0.0001$) (figure 4.18 a). Cells exposed to 60 μM menadione retained 48.76 ± 7.20 % LDH activity in control cells, or 4.91 ± 3.49 % LDH activity in GTG pre-treated cells in comparison ($p < 0.0001$). Control cells retained 9.37 ± 7.82 % LDH activity upon exposure to 80 μM menadione, but cells pre-incubated with GTG showed 100 % cell damage.

Two further experiments confirmed the increased susceptibility of HaCaT cells pretreated with 10 μM compared with control cells at menadione concentrations of 60 μM ($p < 0.01$) and 80 μM to 140 μM ($p < 0.05$) (figure 4.18 b), and at 40 μM, 60 μM, and 120 μM menadione ($p < 0.05$) (figure 4.18 c).

When HaCaT cells were pre-incubated with GTG concentrations of 1, 2.5, 5, 7.5, and 10 μM prior to menadione exposure, there was a tendency towards greater cell damage with increasing GTG concentration (figure 4.19). HaCaT cells exposed to 100 μM menadione were significantly more susceptible to damage when pre-treated with 2.5 μM GTG ($p < 0.05$), 5 μM GTG ($p < 0.001$), 7.5 μM GTG ($p < 0.05$), or 10 μM GTG ($p < 0.01$) compared to control cells exposed to 100 μM menadione but which did not receive GTG pre-treatment. The cells pre-incubated with 2.5 μM GTG suffered significantly more damage ($p < 0.05$) than those treated with 1 μM GTG, and those cells pre-incubated with 5 μM GTG suffered significantly more damage ($p < 0.01$) than those treated with 2.5 μM GTG.

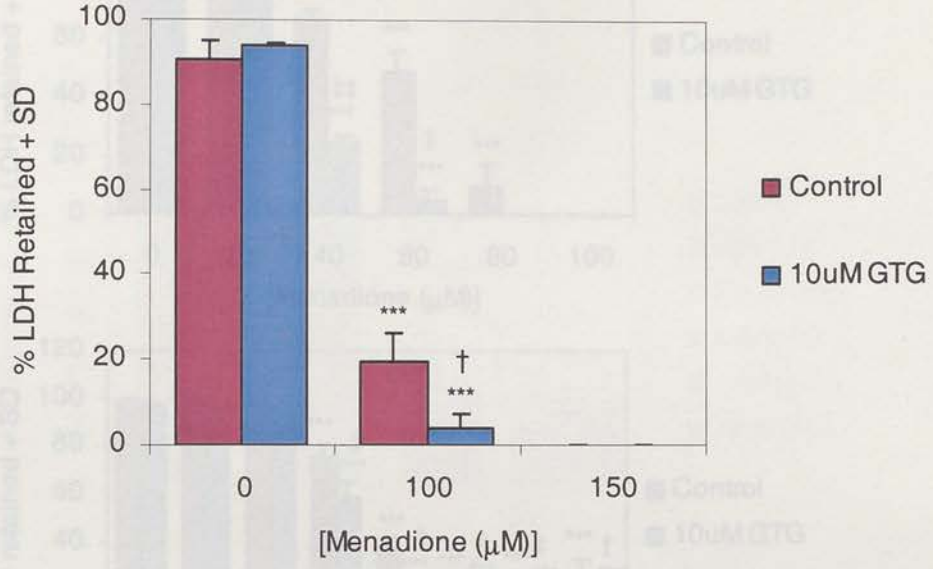


Figure 4.17 The effect of gold thioglucose pre-incubation on the % LDH retained by HaCaT cells exposed to menadione. HaCaT cells were pre-treated with 10 μM gold thioglucose (GTG) for 48 hr prior to exposure to menadione (0, 100, 150 μM) for 18 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.001$*** cf. control cells receiving 0 μM menadione. $p < 0.05$† cf. control cells receiving 100 μM menadione.

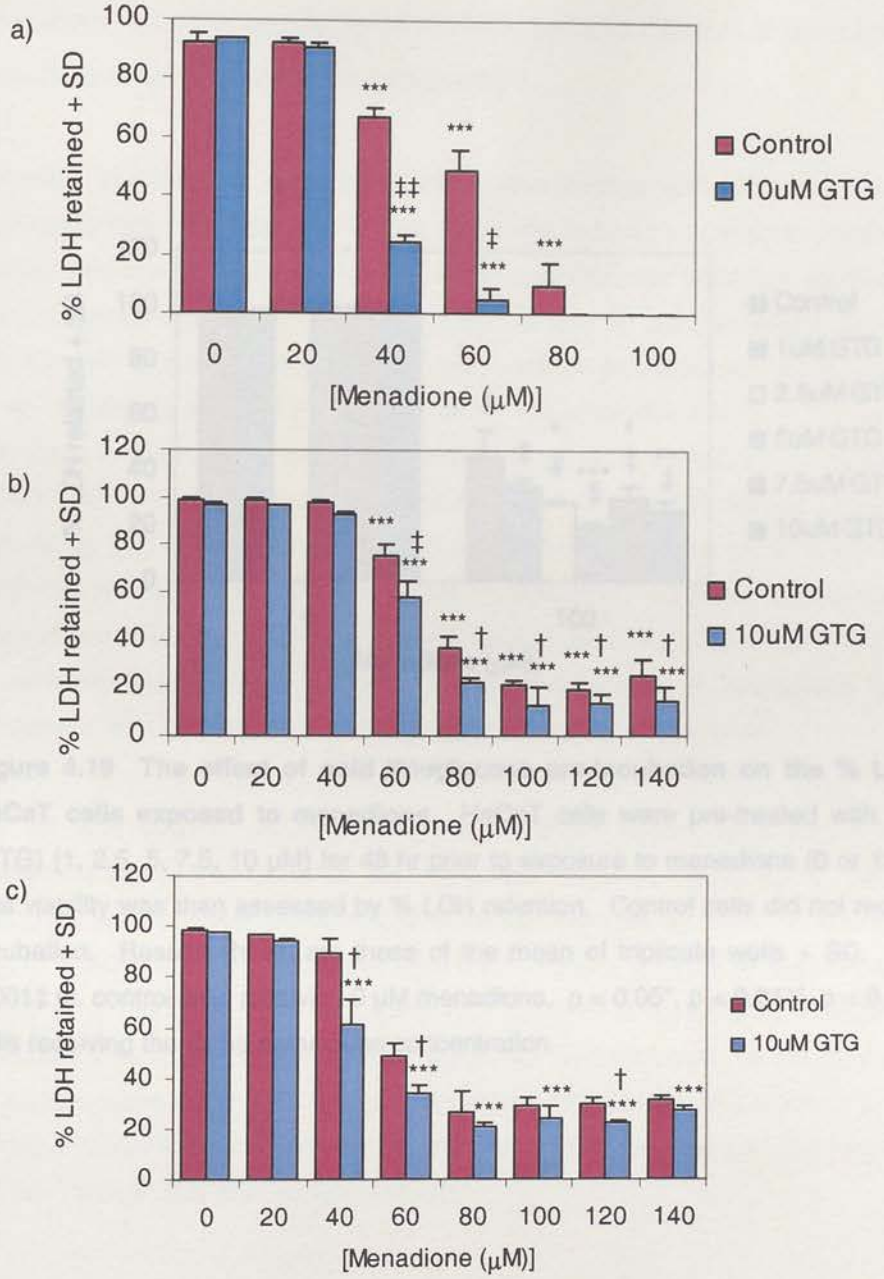


Figure 4.18 The effect of gold thioglucose pre-incubation on the % LDH retained by HaCaT cells exposed to menadione. HaCaT cells were pre-treated with 10μM gold thioglucose (GTG) for 48 hr prior to exposure to menadione (0 to 100 μM; or 0 to 140 μM) for 18 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.001$*** cf. control cells receiving 0 μM menadione. $p < 0.05$†, $p < 0.001$‡, $p < 0.0001$‡‡ cf. control cells receiving the same menadione concentration. Graphs (a) – (c) are each a separate experiment.

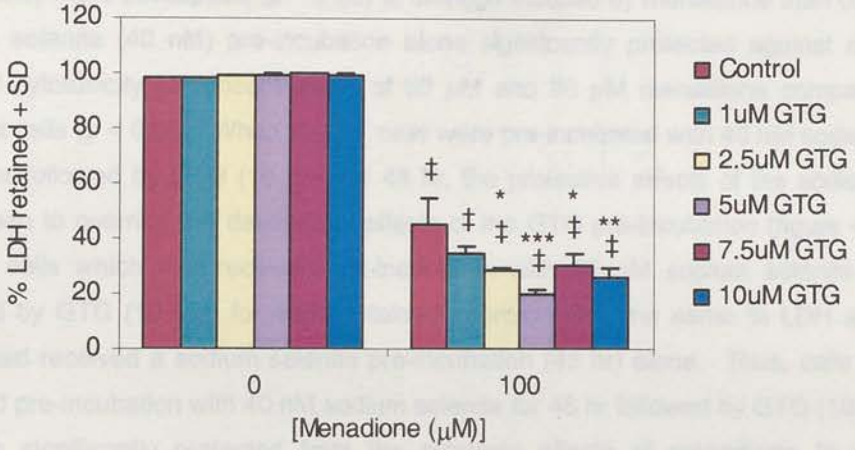


Figure 4.19 The effect of gold thioglucose pre-incubation on the % LDH retained by HaCaT cells exposed to menadione. HaCaT cells were pre-treated with gold thioglucose (GTG) (1, 2.5, 5, 7.5, 10 μM) for 48 hr prior to exposure to menadione (0 or 100 μM) for 18 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.01$††, $p < 0.001$‡ cf. control cells receiving 0 μM menadione. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. control cells receiving the same menadione concentration.

4.3.12 The effect of consecutive sodium selenite and gold thioglucose treatment on susceptibility of HaCaT cells to oxidative damage resulting from menadione exposure

Following exposure to menadione after pre-incubation with 10 μM GTG, cells were significantly more susceptible ($p < 0.05$) to damage induced by menadione than control cells. Sodium selenite (40 nM) pre-incubation alone significantly protected against menadione-induced cytotoxicity at concentrations of 60 μM and 80 μM menadione compared to Se-deficient cells ($p < 0.05$). When HaCaT cells were pre-incubated with 40 nM sodium selenite for 48 hr followed by GTG (10 μM) for 48 hr, the protective effects of the sodium selenite were seen to override the deleterious effects of the GTG pre-incubation (figure 4.20). The HaCaT cells which had received pre-incubation with 40 nM sodium selenite for 48 hr followed by GTG (10 μM) for 48 hr retained approximately the same % LDH as the cells which had received a sodium selenite pre-incubation (48 hr) alone. Thus, cells which had received pre-incubation with 40 nM sodium selenite for 48 hr followed by GTG (10 μM) for 48 hr were significantly protected from the cytotoxic effects of menadione ($p < 0.05$) in comparison with control cells which did not receive any pre-treatment.

The TR and cyGPX activities were measured for HaCaT cells which had received incubations identical to those for the menadione exposure (figure 4.21). The cells which received a GTG (10 μM) pre-incubation alone had significantly lower TR activity (figure 4.21 a) ($p < 0.0001$) and cyGPX activity (figure 4.21 b) ($p < 0.05$) than control cells. Both TR activity ($p < 0.001$) and cyGPX activity ($p < 0.01$) were significantly increased by a pre-incubation with 40 nM sodium selenite compared to control cells. A pre-incubation with 40 nM sodium selenite followed by a pre-incubation with 10 μM GTG resulted in a TR activity and cyGPX activity which were significantly higher ($p < 0.05$, and $p < 0.01$ respectively) than that seen with a GTG pre-incubation alone.

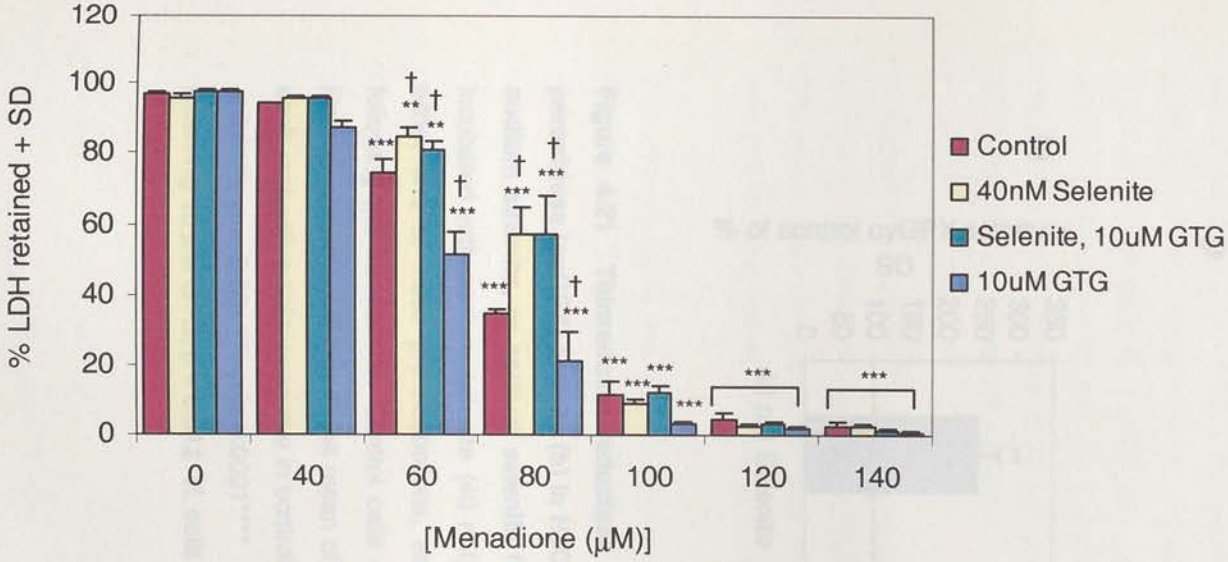


Figure 4.20 The effect of consecutive sodium selenite and gold thioglucose pre-incubations on the % LDH retained by HaCaT cells exposed to menadione. HaCaT cells were pre-treated with sodium selenite (40 nM) followed by gold thioglucose (GTG) (10 μM), or either one of these pre-incubations, each for 48 hr. Cells were washed between and following pre-incubations. Exposure to menadione (0, 40, 60, 80, 100, 120, 140 μM) was for 18 hr. Cell viability was then assessed by % LDH retention. Control cells did not receive a selenite or GTG pre-incubation. Results shown are those of the mean of triplicate wells + SD. $p < 0.01$**, $p < 0.001$*** cf. respective control cells receiving 0 μM menadione. $p < 0.05$† cf. control cells receiving the same menadione concentration.

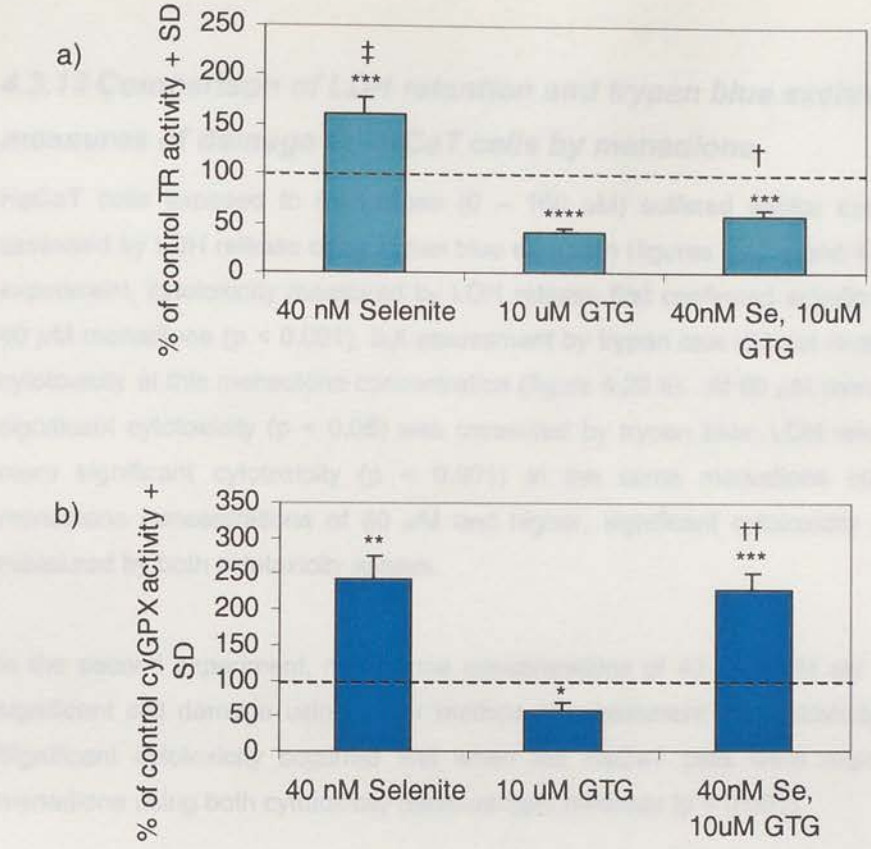


Figure 4.21 Thioredoxin reductase (TR) activity (a), and cytoplasmic glutathione peroxidase (cyGPX) activity (b) in HaCaT cells treated with gold thioglucose (GTG), or sodium selenite, or sodium selenite followed by gold thioglucose. HaCaT cells were incubated with sodium selenite (40 nM) followed by gold thioglucose (GTG) (10 μM), or either one of these pre-incubations, each for 48 hr. Cells were washed between and following pre-incubations. Control cells did not receive a selenite or GTG pre-incubation. Results shown are those of the mean of triplicate flasks + SD. The basal level of activity of each respective selenoenzyme in control cells is indicated by the dashed line. $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, $p < 0.0001$**** cf. control cells; $p < 0.05$† , $p < 0.01$†† cf. cells receiving 10 μM GTG; $p < 0.01$‡ cf. cells receiving 40 nM Se followed by 10 μM GTG.

4.3.13 Comparison of LDH retention and trypan blue exclusion as measures of damage to HaCaT cells by menadione

HaCaT cells exposed to menadione (0 – 160 μM) suffered similar cytotoxicity whether assessed by LDH release or by trypan blue exclusion (figures 4.22 a and 4.22 b). In the first experiment, cytotoxicity measured by LDH release first confirmed significant cytotoxicity at 40 μM menadione ($p < 0.001$), but assessment by trypan blue did not reveal any significant cytotoxicity at this menadione concentration (figure 4.22 a). At 60 μM menadione, slight but significant cytotoxicity ($p < 0.05$) was measured by trypan blue; LDH release measured a more significant cytotoxicity ($p < 0.001$) at the same menadione concentration. At menadione concentrations of 80 μM and higher, significant cytotoxicity ($p < 0.001$) was measured by both cytotoxicity assays.

In the second experiment, menadione concentrations of 40 – 80 μM did not produce any significant cell damage using either method of assessment of cytotoxicity (figure 4.22 b). Significant cytotoxicity occurred first when the HaCaT cells were exposed to 100 μM menadione using both cytotoxicity measurement methods ($p < 0.001$).

LDH release (U/L) has been shown to be almost identical to the corresponding % LDH released (the % released of the total LDH in the cell) (data not shown).

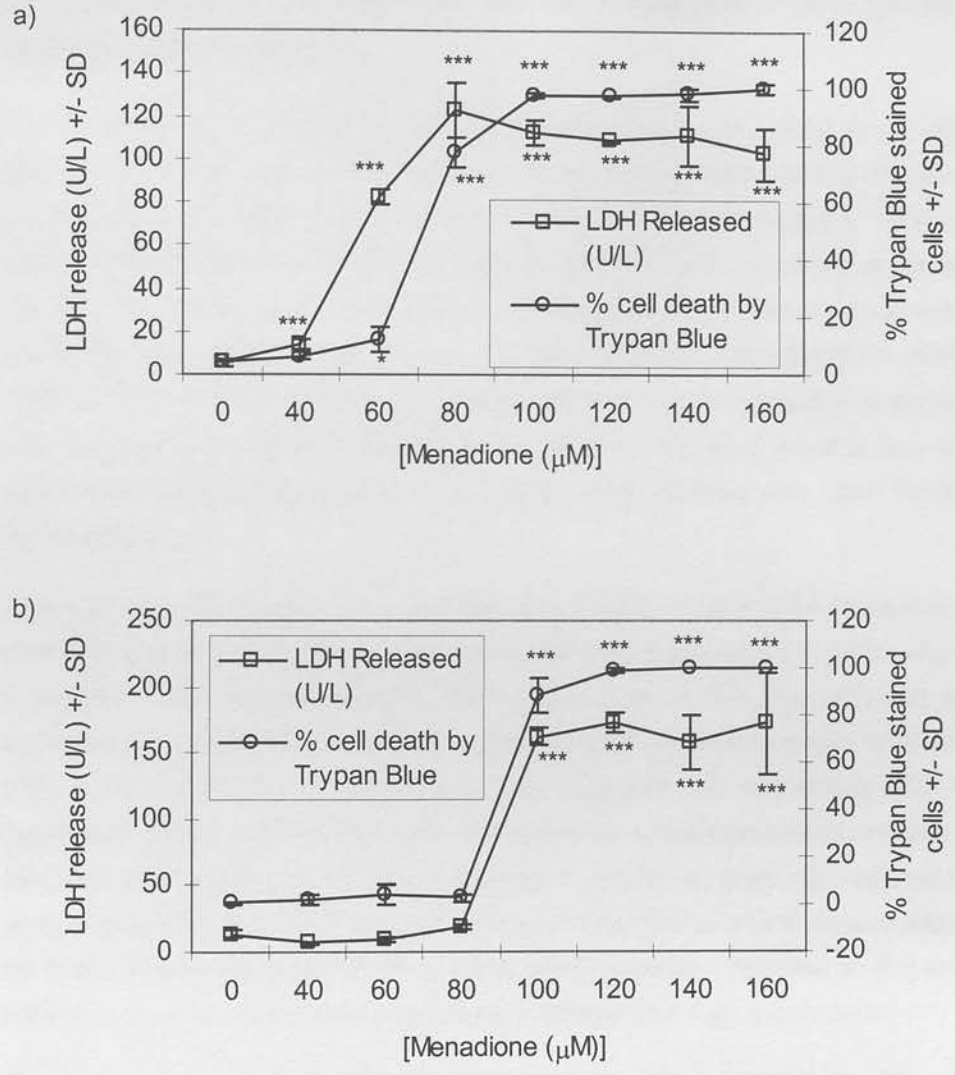


Figure 4.22 Cell viability of HaCaT cells exposed to menadione assessed by LDH release and trypan blue exclusion. Cells were cultured in Se-deficient medium, and incubated with a range of concentrations of menadione (0 – 160 μM) for 18 hr. Cell medium was assayed for LDH activity, and the cells were counted for trypan blue staining. Results shown are those of the mean of triplicate wells ± SD. Where the SD are not visible, they lie within the data point. $p < 0.05$*, $p < 0.001$*** cf. control cells exposed to 0 μM menadione. Graphs (a) and (b) are each a separate experiment.

4.3.14 Assessment of LDH retention as a measure of damage to HaCaT cells by UVB irradiation

When cell damage mediated by the varying UVB doses was plotted as % LDH retained (figure 4.23), it was apparent that the apparent cell damage even with the highest UVB dose did not agree with experiments assessed by trypan blue in the literature. LDH retention by HaCaT cells measured 48 hr after exposure to 1200 J/m^2 UVB was measured at 62.9 ± 6.1% (i.e. 37.1 % 'cell damage'). Cell damage measured by trypan blue assay at a dose of 960 J/m^2 UVB has previously been measured at 69 % (Rafferty, 2000) and 80 % (Rafferty *et al.*, 1998) in HaCaT. This discrepancy between different cytotoxicity assays suggested that the LDH may have been denatured/inactivated by the UVB; therefore the data from three further experiments was plotted as total LDH activity (LDH released plus LDH retained in U/L) (figure 4.24 a - c).

Total LDH was decreased in a dose-dependent manner by UVB when measured 24 hr after irradiation (figure 4.24 a). No inactivation of LDH was apparent at 6 or 12 hr post-irradiation. In another experiment extending to 48 hr (figure 4.24 b), the total LDH was significantly decreased by a UVB dose of 1200 J/m^2 when the LDH measurement was taken at 12 hr ($p < 0.05$) or 24 hr ($p < 0.001$) post-irradiation when compared with cells at time zero. In the third experiment (figure 4.24 c), the LDH decreased in a dose-dependent manner with UVB irradiation when measured 48 hr post-irradiation. Whilst this trend was also apparent for all other time points used, there was an increase in total LDH at a UVB dose of 480 J/m^2 when the LDH measurement was 72, 96 or 120 hr post-irradiation. This may be due to a resistant population of cells or uninjured cells having multiplied over such a time period.

There was high variability in the total LDH between the three experiments. The cells used to produce the data illustrated in graph 4.24 c may have been pre-confluent, since the total LDH had increased by 24 hr; this was not apparent in the other two graphs. The cells may have responded differently to the cytotoxic insult due to differences in their stage of growth. The data in graph 4.24 c is also different to that of graphs 4.24 a and 4.24 b since the LDH values of the control are 500 U/L, compared to 300 U/L total LDH in graphs a and b. There is no clear explanation for this discrepancy, but it further illustrates potential problems in using LDH as a measure of cytotoxicity resulting from *in vitro* exposure of cells to UVB. It is also unclear why the amount of total LDH increased with increasing UVB dose in the cells receiving 0 J/m^2 UVB in graph 4.24 b. This may be due to methodological errors.

The inactivation of LDH did not appear to be immediate since the levels of LDH in sham-irradiated control cells and cells which were irradiated and assayed for LDH immediately had similar levels of LDH.

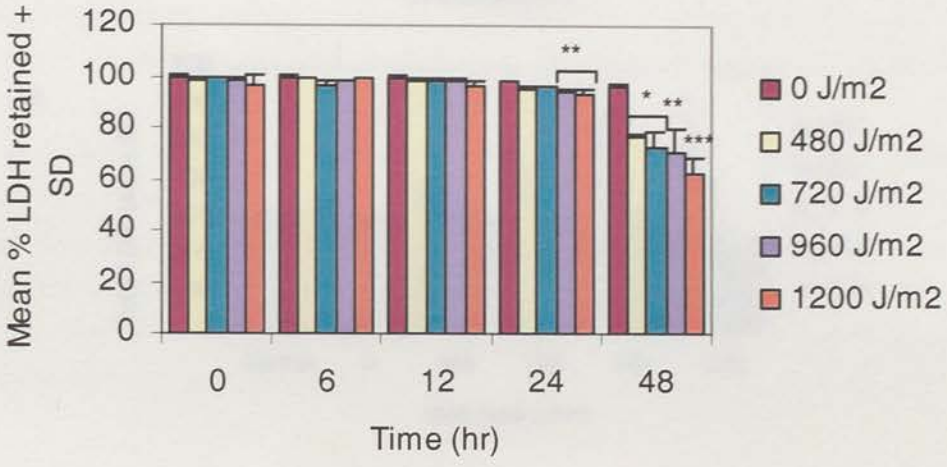


Figure 4.23 The effect of UVB irradiation on the % LDH retained by HaCaT cells after 0, 6, 12, 24 or 48 hr. HaCaT cells were irradiated with UVB (480 – 1200 J/m^2) through PBS, and the original medium replaced on the cells for 0, 6, 12, 24, or 48 hr before LDH assay took place. Results shown are those of the mean of triplicate wells + SD. $p < 0.05$*, $p < 0.01$**, $p < 0.001$*** cf. control cells, at the respective time point, which did not undergo irradiation with UVB (represented by 0 J/m^2 UVB).

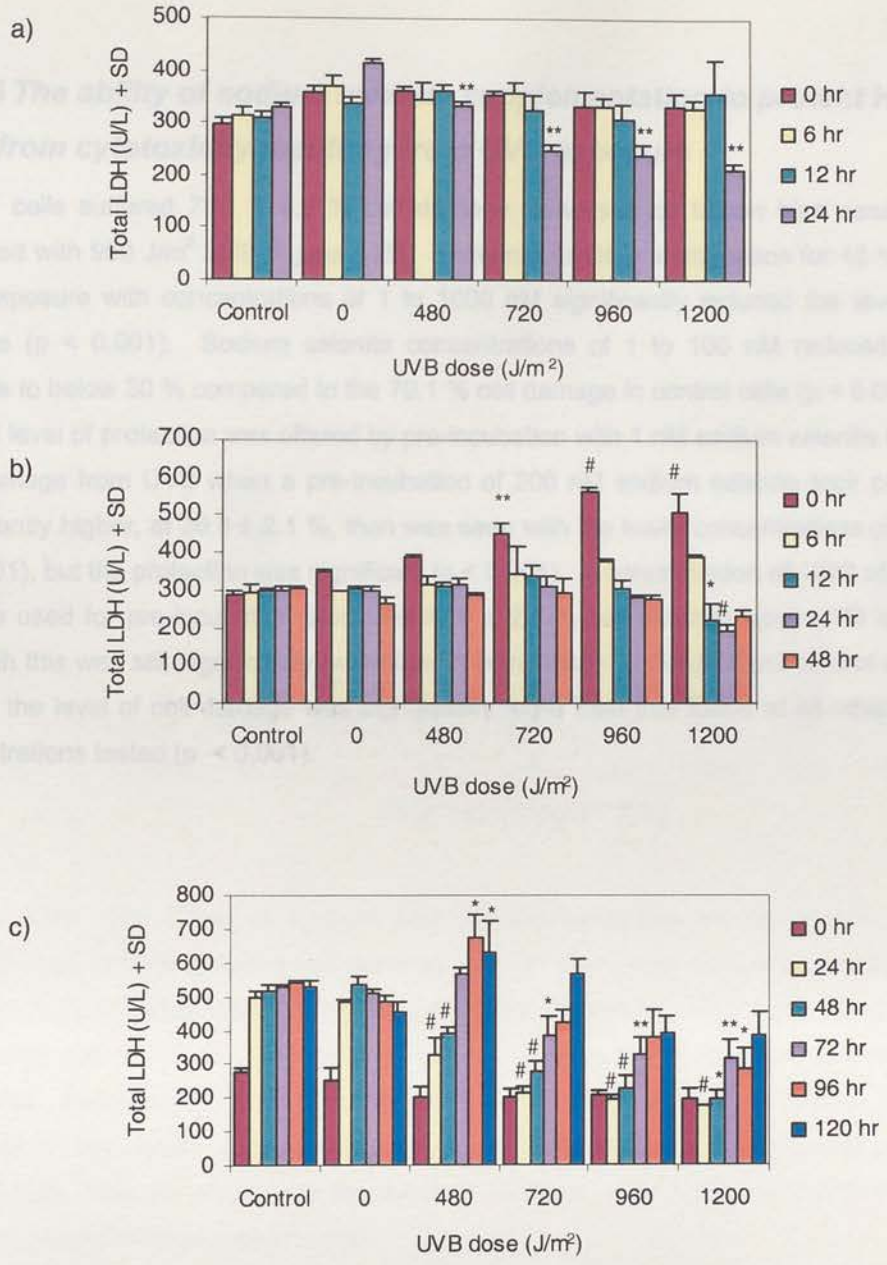


Figure 4.24 The effect of UVB irradiation on the total LDH of HaCaT cells. HaCaT cells were irradiated with UVB (480 – 1200 J/m^2) through PBS, and the original medium replaced on the cells for 0, 6, 12, 24, 48, 72, 96 or 120 hr before LDH assay took place. Results shown are those of the mean of triplicate wells + SD. Total LDH was calculated by addition of LDH released and retained (U/L). $p < 0.05$*, $p < 0.01$**, $p < 0.001$# cf. cells which were irradiated and had their LDH assessed immediately (time zero). Control cells were sham irradiated. Graphs (a) – (c) are each a separate experiment.

4.3.15 The ability of sodium selenite supplementation to protect HaCaT cells from cytotoxicity resulting from UVB exposure

HaCaT cells suffered 79.1 ± 4.2 % cell damage, assessed by trypan blue assay, when irradiated with 960 J/m^2 UVB (figure 4.25). Sodium selenite pre-incubation for 48 hr prior to UVB exposure with concentrations of 1 to 1000 nM significantly reduced the level of cell damage ($p < 0.001$). Sodium selenite concentrations of 1 to 100 nM reduced the cell damage to below 30 % compared to the 79.1 % cell damage in control cells ($p < 0.001$). The optimal level of protection was offered by pre-incubation with 1 nM sodium selenite for 48 hr. Cell damage from UVB when a pre-incubation of 200 nM sodium selenite took place was significantly higher, at 36.0 ± 2.1 %, than was seen with the lower concentrations of selenite ($p < 0.01$), but the protection was significant ($p < 0.001$). A concentration of 1000 nM sodium selenite used for pre-incubation produced 62.0 ± 2.1 % cell damage upon UVB exposure; although this was still significantly protective in comparison to Se-deficient control cells ($p < 0.001$), the level of cell damage was significantly more than that found at all other selenite concentrations tested ($p < 0.001$).

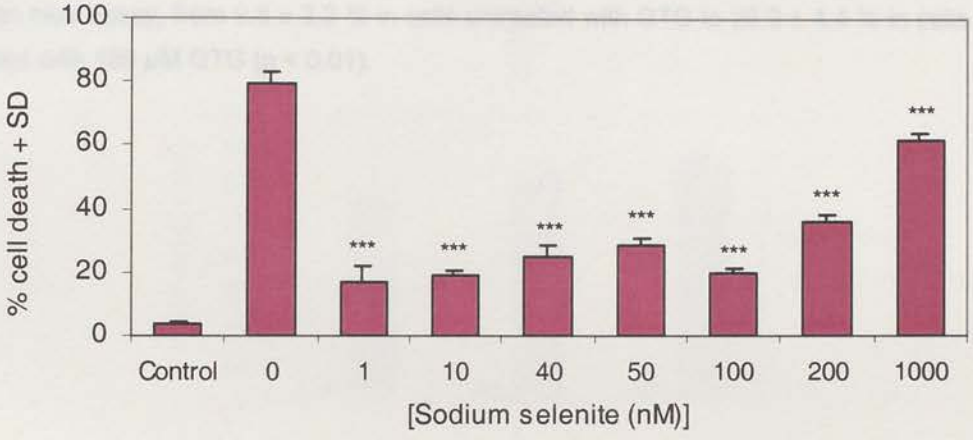


Figure 4.25 The effect of sodium selenite pre-incubation on the susceptibility of HaCaT cells to UVB-induced cell damage. HaCaT cells were incubated with Se-deficient medium supplemented with different concentrations of sodium selenite (1, 10, 40, 50, 100, 200, 1000 nM) for 48 hr. Control cells received no Se supplementation, and were sham-irradiated. Following the pre-incubation the HaCaT cells were exposed to UVB (960 J/m^2). After 48 hr cell viability was assessed by determining the % of cells staining positive for trypan blue. Results shown are the mean of triplicate wells + SD. $p < 0.001^{***}$ cf. Se-deficient control cells exposed to UVB.

4.3.16 The ability of gold thioglucose supplementation to modify the susceptibility of HaCaT cells to cytotoxicity resulting from UVB exposure

Pre-incubation of HaCaT cells with either 1 μM or 10 μM GTG prior to an irradiation with 720 J/m^2 UVB did not affect the % cell damage in any of three separate experiments (figure 4.26). In the third experiment (figure 4.26 c), a pre-incubation of HaCaT cells with 100 μM GTG for 48 hr prior to irradiation significantly increased the % cell damage, as measured by trypan blue assay, from 9.8 ± 3.3 % in cells untreated with GTG to 28.2 ± 4.4 % in cells pre-treated with 100 μM GTG ($p < 0.01$).

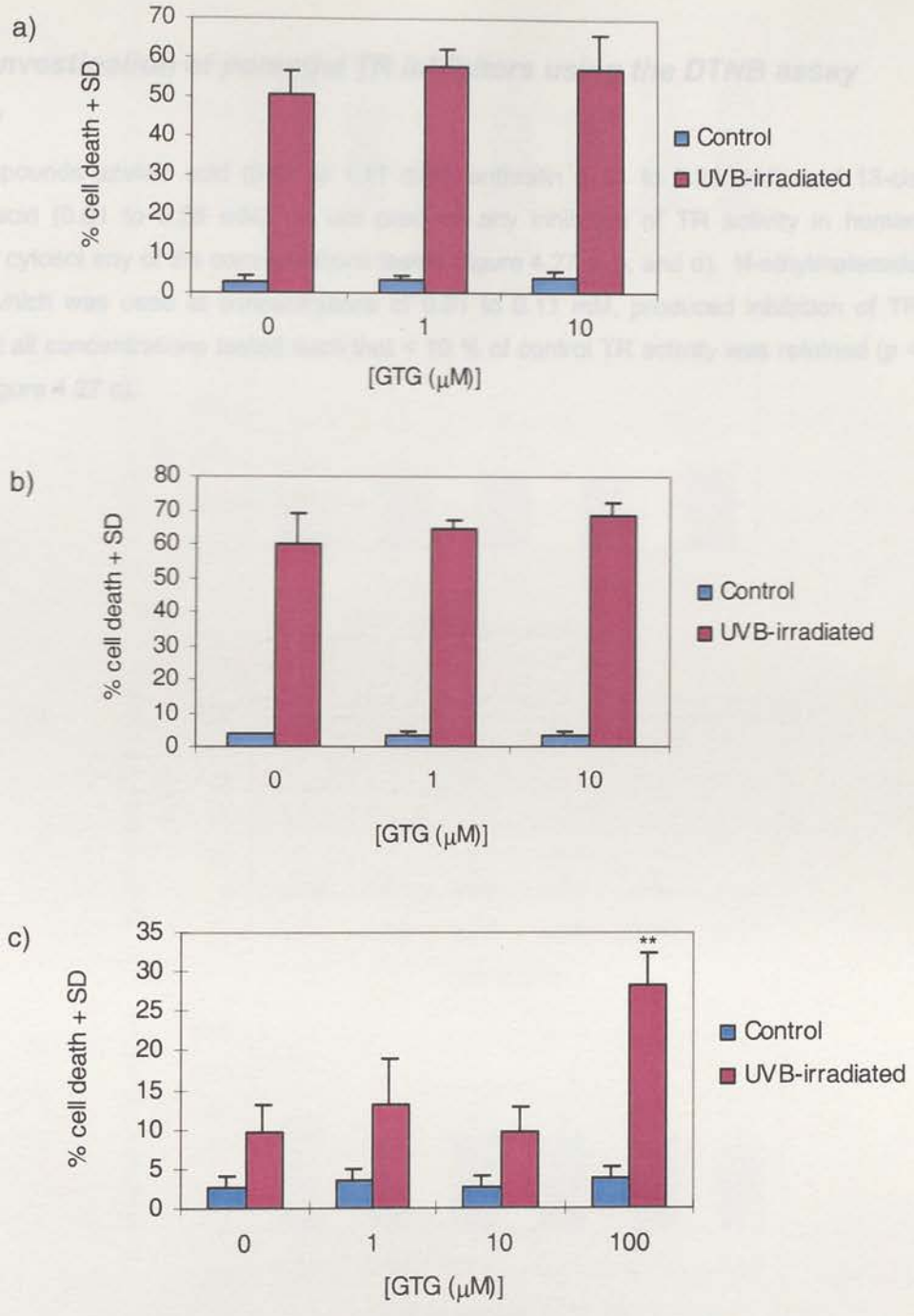


Figure 4.26 The effect of gold thioglucose pre-incubation on UVB-mediated cell damage of HaCaT cells. HaCaT cells were pre-treated with 1, 10, or 100 μM gold thioglucose (GTG) for 48 hr prior to exposure to UVB-irradiation (720 J/m^2). Cell viability was assessed 48 hr later by trypan blue assay. Control cells did not receive UVB-irradiation. Results shown are those of the mean of triplicate wells + SD. $p < 0.01$** cf. UVB-irradiated control cells receiving 0 μM GTG pre-incubation. Graphs (a) – (c) each represent a separate experiment.

4.3.17 Investigation of potential TR inhibitors using the DTNB assay system

The compounds azelaic acid (0.01 to 1.11 mM), anthralin (0.01 to 0.28 mM), and 13-cis retinoic acid (0.01 to 0.56 mM) did not produce any inhibition of TR activity in human placental cytosol any of the concentrations tested (figure 4.27 a, b, and d). N-ethylmalemide (NEM), which was used at concentrations of 0.01 to 0.11 mM, produced inhibition of TR activity at all concentrations tested such that < 10 % of control TR activity was retained ($p < 0.001$) (figure 4.27 c).

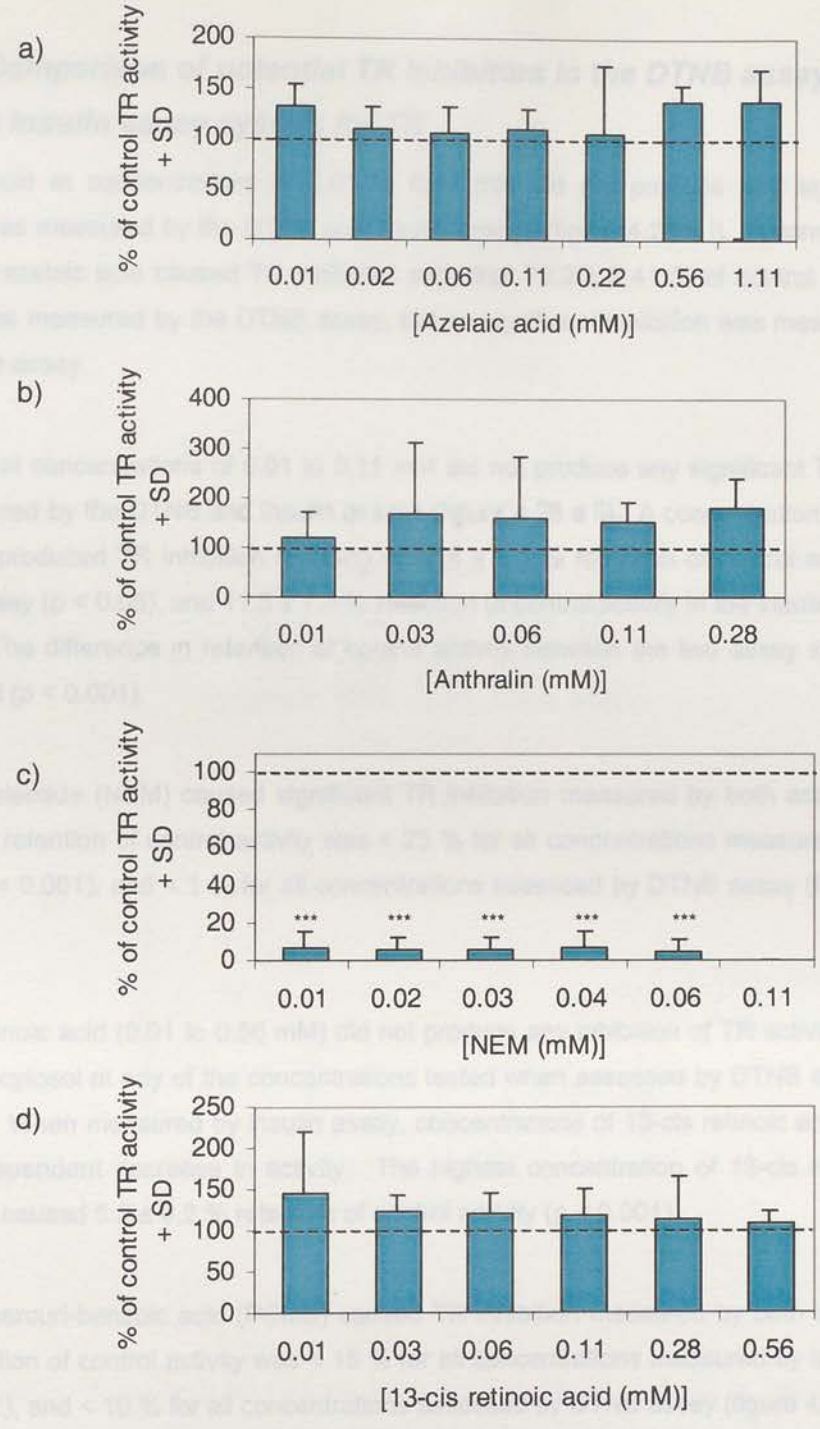


Figure 4.27 The effect of various TR inhibitors on TR activity in human placental cytosol assessed by DTNB assay. The results shown are the meaned data from two experiments. The inhibitors assessed were (a) azelaic acid; (b) anthralin; (c) N-ethylmaleimide (NEM); and (d) 13-cis retinoic acid. The inhibitor concentrations quoted are final concentrations in the assay. Incubation of placental cytosol with the inhibitors took place overnight at 4°C. $p < 0.001$*** cf. control TR activity.

4.3.18 Comparison of potential TR inhibitors in the DTNB assay system and the insulin assay system for TR

Azelaic acid at concentrations of 0.01 to 0.56 mM did not produce any significant TR inhibition as measured by the DTNB and insulin assays (figure 4.28 a i). A concentration of 1.11 mM azelaic acid caused TR inhibition such that 79.9 ± 2.41 % of control activity was retained as measured by the DTNB assay, but no significant inhibition was measured using the insulin assay.

Anthralin at concentrations of 0.01 to 0.11 mM did not produce any significant TR inhibition as measured by the DTNB and insulin assays (figure 4.28 a ii). A concentration of 0.28 mM anthralin produced TR inhibition resulting in 87.4 ± 3.2 % retention of control activity in the DTNB assay ($p < 0.05$), and 11.5 ± 7.8 % retention of control activity in the insulin assay ($p < 0.001$). The difference in retention of control activity between the two assay systems was significant ($p < 0.001$).

N-ethylmalemide (NEM) caused significant TR inhibition measured by both assay systems such that retention of control activity was < 25 % for all concentrations measured by insulin assay ($p < 0.001$), and < 1 % for all concentrations assessed by DTNB assay (figure 4.28 a iii).

13-cis retinoic acid (0.01 to 0.56 mM) did not produce any inhibition of TR activity in human placental cytosol at any of the concentrations tested when assessed by DTNB assay (figure 4.28 b i). When measured by insulin assay, concentrations of 13-cis retinoic acid produced a dose-dependent decrease in activity. The highest concentration of 13-cis retinoic acid, 0.56 mM, caused 5.2 ± 0.2 % retention of control activity ($p < 0.001$).

p-chloromercuri-benzoic acid (PCMB) caused TR inhibition measured by both assays such that retention of control activity was < 15 % for all concentrations measured by insulin assay ($p < 0.001$), and < 10 % for all concentrations assessed by DTNB assay (figure 4.28 b ii).

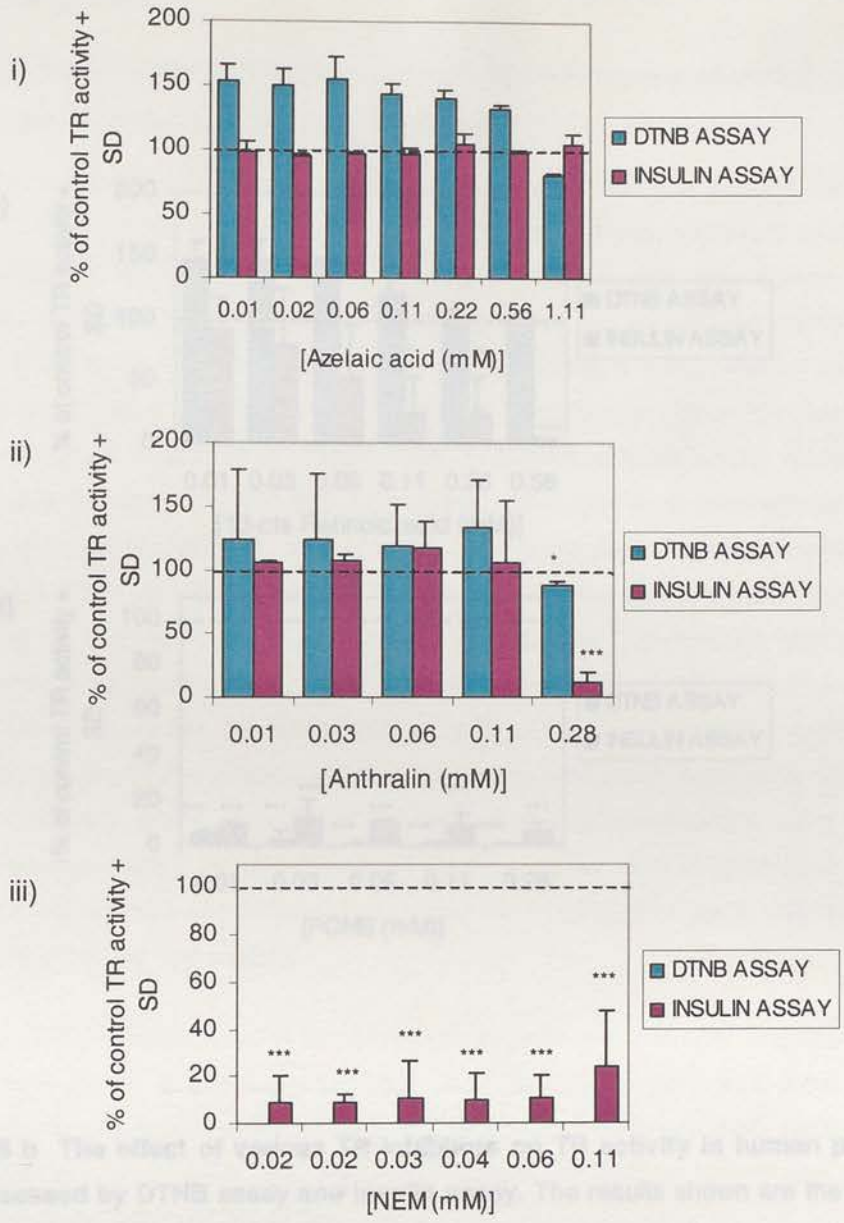


Figure 4.28 a The effect of various TR inhibitors on TR activity in human placental cytosol assessed by DTNB assay and insulin assay. The results shown are the meaned data from two experiments. The inhibitor concentrations quoted are final concentrations in the assay. Incubation of placental cytosol with the inhibitors took place overnight at 4˚C. $p < 0.05$*; $p < 0.001$*** cf. control activity. The TR activity of the control is indicated by the dashed line.

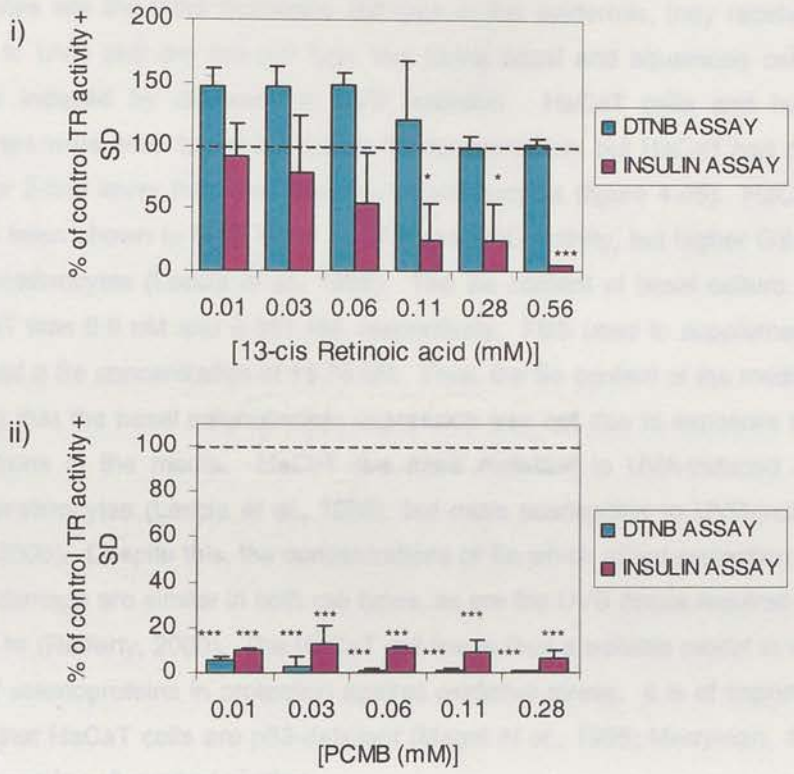


Figure 4.28 b The effect of various TR inhibitors on TR activity in human placental cytosol assessed by DTNB assay and insulin assay. The results shown are the meaned data from two experiments. The inhibitor concentrations quoted are final concentrations in the assay. Incubation of placental cytosol with the inhibitors took place overnight at 4˚C. $p < 0.001$*** cf. control activity. The TR activity of the control is indicated by the dashed line.

4.3 DISCUSSION

The HaCaT cell line as a model of human keratinocytes in which to study oxidative damage and selenoproteins

Keratinocytes are the most numerous cell type in the epidermis, they receive the highest exposure to UVB and are the cell type that forms basal and squamous cell carcinomas, which are induced by exposure to UVB radiation. HaCaT cells and human primary keratinocytes were seen to have a similar TR concentration, but HaCaT had cyGPX activity of an order 2-fold lower than that of primary keratinocytes (figure 4.05). HaCaT cells have previously been shown to have lower cyGPX and SOD activity, but higher GSH levels, than primary keratinocytes (Leccia *et al.*, 1998). The Se content of basal culture media for PK and HaCaT was 8.9 nM and 0.351 nM, respectively. FBS used to supplement the HaCaT medium had a Se concentration of 13.78 nM. Thus, the Se content of the media was similar, suggesting that the basal selenoprotein expression was not due to exposure to differing Se concentrations in the media. HaCaT are more resistant to UVA-induced damage than primary keratinocytes (Leccia *et al.*, 1998), but more susceptible to UVB-induced damage (Rafferty, 2000). Despite this, the concentrations of Se which afford protection against UVB-mediated damage are similar in both cell types, as are the UVB doses required to kill 70 % of cells at 48 hr (Rafferty, 2000). The HaCaT cell line is thus a suitable model in which to study the role of selenoproteins in protection against oxidative stress. It is of importance to note, however, that HaCaT cells are p53-deficient (Magal *et al.*, 1998; Merryman, 1999) and this limits their use in cell survival studies.

The doses of UVB irradiation used in the cytotoxicity experiments (720 J/m^2 and 960 J/m^2) in this thesis were similar to physiological doses of UVB. The physiological dose of UVB required to cause mild reddening of the skin, i.e. the standard erythemal dose (SED), is 150 J/m^2 for human skin type I (very pale, always burns) and 600 J/m^2 for type IV skin (olive or mediterranean complexion) (Honigsmann, 2002).

The Trypan blue and LDH assay systems for assessing cytotoxicity

Cell damage was assessed in all the menadione cytotoxicity experiments using LDH retention, and in the UV experiments using trypan blue exclusion since LDH was unsuitable as a measure when UVB was the stressor. Exposure to UVB inactivated LDH in a dose-dependent manner in HaCaT cells (figure 4.24). Previous studies have demonstrated that UV can inactivate LDH both *in vitro* (Artiukhova *et al.*, 1997; Chen *et al.*, 1989) and *in vivo* (Löfgren and Söderberg, 2001), in a dose-dependent manner (Chen *et al.*, 1989) consistent with the results presented here. The damage to LDH by UV appears to be ROS-mediated

since irradiation in the presence of ROS scavengers/quenchers results in restoration of catalytic activity (Artiukhova *et al.*, 1997). The absorption spectrum of LDH changes during UVB exposure, suggesting an alteration of tryptophan residues in the LDH molecule (Chen *et al.*, 1989). Photo-oxidation of tryptophan also causes oxidation of tyrosine, which may alter the structural conformation of the enzyme, and possibly its activity. The loss of LDH activity is due to decreased activity of the LDH molecules rather than an altered affinity between the enzyme and substrate (Chen *et al.*, 1989).

Previous studies have shown the LDH assay and trypan blue assay correlate well in their assessment of cytotoxicity in rat hepatocytes (R = 0.92) (Jauregui *et al.*, 1981). Our studies suggest that the two methods correlate well in the assessment of menadione cytotoxicity in HaCaT cells.

Cellular confluence as a source of variation in cytotoxicity

The damage suffered by HaCaT cells upon exposure to menadione was influenced by cellular confluence level (figures 4.09, 4.10, 4.11). Pre-confluent cells were more susceptible to damage ($p < 0.01$) by menadione than were 100 % confluent cells, with post-confluent cells the most resistant to damage. These results are similar to those obtained with EAhy926 cells (section 3.4). In HaCaT cells the expression of keratinocyte growth factor receptors is induced by confluence and high cell density (Capone *et al.*, 2000). Such expression of growth factor receptors may have concomitant effects on the ability of cells to resist oxidative stress. The levels of cyGPX, PHGPX, and TR activity and concentrations did not increase with increasing confluency in HaCaT cells (figure 4.12). Thus, it does not appear that the increased resistance to damage with confluency level was due to increases in selenoenzyme expression. However, this was a single experiment only, so the results need to be confirmed in further studies. The increased resistance may be due to other antioxidant enzymes that we did not measure. Other possible explanations for the differences in susceptibility with confluency level are discussed in section 3.4. For all further experiments, HaCaT cells were treated with menadione or UVB when at 100 % confluence to reduce the variation between experiments.

Protection against menadione- and UVB-mediated damage by Se, and Se supplementation of skin cells

Se-deficient HaCaT cells pre-incubated with sodium selenite (1 to 1000 nM) for 48 hr were significantly less sensitive to damage by menadione ($p < 0.01$) (Figure 4.13). Optimal protection against menadione-induced cytotoxicity ($p < 0.01$) was offered by 10 nM sodium selenite in two out of three experiments (figure 4.13 a, b). Protection was not lost at higher selenite concentrations. Some of the variation observed between the individual experiments

was likely to be due to confluence differences since some experiments were performed using non-confluent cells.

Pre-incubation with sodium selenite, inducing selenoprotein expression, was necessary for protection against menadione cytotoxicity (figure 4.14). Sodium selenite added to the cells at the same time as menadione was possibly acting as a pro-oxidant, overwhelming the antioxidant capacity of the cells with oxidative stress resulting from insults from both menadione and sodium selenite simultaneously. One of these compounds may be reduced or possibly redox-cycled by the other, causing formation of further ROS.

Pre-incubation of HaCaT cells for 48 hr with sodium selenite (1 to 1000 nM) provided significant protection ($p < 0.001$) from cytotoxic insult by UVB irradiation as assessed by trypan blue assay (figure 4.25). Optimal protection was offered by 1 nM sodium selenite ($p < 0.001$). Loss of protection was seen with increasing concentrations of sodium selenite greater than 100 nM such that at 1000 nM no protective effect of selenite was observed. Such loss of protection was shown previously in primary human keratinocytes and HaCaT (Rafferty *et al.*, 1998a). Loss of protection was associated with a loss of cyGPX activity ($p < 0.05$), but not of TR or PHGPX expression (see below). When menadione was used as the oxidative stressor, no loss of protection was demonstrated at the higher selenite concentrations.

Se-deficient HaCaT cells supplemented with sodium selenite demonstrated dose-dependent increases in TR activity. TR activity was optimal at 10 nM sodium selenite ($p < 0.05$, $p < 0.001$). Increasing the selenite concentration above 10 nM did not further increase the TR activity. This was not the case with cyGPX activity. The cyGPX activity of HaCaT cells decreased with increasing selenite concentration. At 1000 nM selenite, the cyGPX activity was not significantly different to Se-deficient controls. One explanation for this decrease in cyGPX activity could be that ROS are being produced by sodium selenite in the cell, and although the level of ROS is not enough to cause membrane damage and LDH release, the ROS are at a level substantial enough to inactivate or destroy cyGPX. The activity of cyGPX can be induced or inactivated by oxidative stress, which may be dependent upon the level of the stress. Enzymatic inactivation of cyGPX by ROS has been demonstrated in cultured keratinocytes (Vessey and Lee, 1993). Some enzymes may be more susceptible to oxidative inactivation because the active site is more accessible to ROS, or because they contain functional groups consisting of amino acids which are more easily damaged. ROS react with several amino acid residues *in vitro*, generating modified and less active enzymes, cross-linked, denatured, or inactive proteins. Among the most susceptible amino acids are sulphur- (or selenium)-containing residues, methionine and cysteine residues (Halliwell and Gutteridge, 1999). Such loss of cyGPX activity was not seen in EAhy926 cells with these

selenite concentrations, and this mechanism of ROS-induced inactivation would likely be common to cell types. EAhy926 cells have a higher amount of TR than skin cells however, so potential ROS could be reduced before they had an opportunity to inactivate cyGPX in endothelial cells.

An alternative explanation for the down-regulation of cyGPX may be end product inhibition involving negative feedback of selenide, which down-regulates selenoenzyme expression at high sodium selenite concentrations. Sustained exposure (weeks) of cells to high levels of Se, generating reactive Se intermediates may cause diselenide formation, leading to inhibition of TR activity over time (Ganther, 1999). It would seem a reasonable assumption that the same may be true for inhibition of GPX activity by diselenide formation with the SeCys of its active site. However, incubation with selenite was only for 48 hr in our experiments, which may be insufficient time for such inhibition to occur. TR and cyGPX can be regulated independently, for example, p53 expression results in elevated cyGPX expression, but down-regulation of TR expression (Gladyshev *et al.*, 1998). The inhibition of GPX activity in skin cells, but lack of inhibition of TR activity observed in the studies presented here may represent selenide formation which differs in its threshold for effects on TR and GPX. However, recent studies have revealed that methylseleninate, a metabolite of Se, does not inhibit TR activity, but is a substrate for the enzyme (Gromer and Gross, 2002). Thus, the mechanism is far from clear.

The loss of protection appears not be to due to cytotoxicity of sodium selenite, since concentrations up to 1000 nM were not cytotoxic to HaCaT cells, as assessed by LDH release and gross morphological examination by light microscope. However, the pro-oxidant status of sodium selenite at μM concentrations is well documented in both *in vivo* and *in vitro* studies (Dougherty and Hoekstra, 1982; Rafferty *et al.*, 1998a; Stewart *et al.*, 1999; Yan and Spallholz, 1993), and 1000 nM selenite has previously been shown to be toxic to HaCaT cells as assessed by trypan blue assay (Rafferty, 2000). The LDH and trypan blue assay systems, which correlate well in their assessment of cytotoxicity, are discussed in earlier sections. The reason for the apparent discrepancies in cytotoxic measurements between studies are unclear. One possible explanation is differing sensitivity between the two assay systems.

Sodium selenite can oxidize glutathione to form selenodiglutathione (GSSeSG), which is cytotoxic to cultured tumour cell lines, and other thiols to form selenotrisulphides which react to produce $O_2^{\bullet-}$ and H_2O_2 (Spallholz, 1994). At higher selenite concentrations, GSSeSG may be formed, causing GSH depletion which could also account for loss of cyGPX activity.

Supplementation of Se-deficient HaCaT cells with selenomethionine gave optimal TR and PHGPX activities at 1000 nM selenomethionine ($p < 0.05$), and cyGPX at 200 nM selenomethionine ($p < 0.01$) (figure 4.03). In the second experiment, much higher concentrations were needed to induce all three selenoenzymes (figure 4.04). At a concentration of 100,000 nM selenomethionine there was no significant difference in the cyGPX activity compared to the basal level in control cells. LDH release, and gross morphological examination by light microscope, did not indicate any cytotoxicity to HaCaT cells at any selenomethionine concentration tested.

Using selenomethionine concentrations from 10 nM to 1000 nM, TR, PHGPX and cyGPX activities of HaCaT cells were optimal at 1000 nM. At this selenomethionine concentration, Rafferty *et al.* saw a decrease in the protection of HaCaT cells from UVB, but the reason for this decrease in unclear. Selenomethionine did not show any signs of cytotoxicity to HaCaT cells up to a concentration of 100 μM in the studies presented here, as assessed by LDH release. Concentrations of up to 10 μM selenomethionine show no cytotoxicity to primary human keratinocytes as assessed by trypan blue exclusion (Rafferty *et al.*, 1998a). It would appear that the loss of protection was not due to cytotoxicity of the selenomethionine or to a decrease in selenoenzyme expression. The loss of protection demonstrated with selenomethionine may be due to a mechanism distinct from that operating with sodium selenite.

Supplementation of HaCaT cells with Se in the form of selenomethionine produced increases of a lesser magnitude in TR and GPX activity than those produced by sodium selenite. A concentration of 100 nM sodium selenite increased TR activity by 3.7-fold and 1.8-fold respectively in two separate experiments (figures 4.01 and 4.02), whereas 100 nM selenomethionine increased TR activity in HaCaT by 1.3-fold in two experiments (figures 4.03 and 4.04). Although these data cannot be directly compared as assays were performed on different batches of cells at different times, it suggests that the organic form of Se, selenomethionine, induces TR expression to a lesser extent than sodium selenite, a non-organic form. Berggren *et al.* have also shown that L-selenomethionine produces a smaller change in TR activity than sodium selenite (Berggren *et al.*, 1997). The chemical form of Se added to cells is an important factor in determining the ability of Se to afford protection from UVB irradiation in skin cells (Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b). Optimal protection of human primary keratinocytes was afforded by 10 nM sodium selenite, and by 50 nM selenomethionine, demonstrating the lesser potency of selenomethionine. Studies using endothelial cells have also shown that sodium selenite is more potent than selenomethionine in conferring protection against t-BuOOH-mediated cytotoxicity in cultured

HUVEC. This difference may reflect the differing abilities of each compound to modify selenoprotein status.

L-methionine is present in different culture media (DMEM medium for HaCaT cells: 30 mg/L), and may compete with the selenomethionine for its Se. This may explain the lower efficacy of selenomethionine in the protection of HUVEC against toxicity from t-BuOOH, and the protection of keratinocytes against UVB-irradiation. It is feasible that there is less selenomethionine uptake by the cell when methionine is competing, resulting in less bioactivity and efficacy. The degree of selenomethionine incorporation into proteins depends upon the dosage and methionine status, and diminishes at high methionine intakes (Schrauzer, 2001). Methionine-free culture media could be used to investigate this. However, preliminary experiments with methionine-free medium have demonstrated that cells in culture do not grow well in such medium (personal communication, Dr Forbes Howie).

Studies in skin cells using GTG to examine the role of individual selenoproteins in protection

Se was demonstrated to protect against cytotoxicity in HaCaT cells mediated by UVB and menadione, but it was essential to determine which selenoenzymes were responsible for the protection. To investigate this, the differing sensitivities of the three selenoenzymes to inhibition by gold thioglucose (GTG) were exploited.

After pre-incubation with 10 μM GTG (18.1 % of TR activity ($p < 0.001$), 93.0 % of cyGPX activity, and 71.1 % of PHGPX activity retained), HaCaT cells were significantly more susceptible ($p < 0.05$) to damage by menadione (figures 4.17, 4.18, 4.19, 4.20). This would suggest that TR is important in the protection of HaCaT cells from oxidative stress mediated by menadione.

Pre-incubation of HaCaT cells with 10 μM GTG prior to an irradiation with 720 J/m^2 UVB did not affect the extent of cell damage (figure 4.27). Pre-incubation of HaCaT cells with 100 μM GTG (3.18 % TR activity ($p < 0.001$) 33.3 % cyGPX activity ($p < 0.001$) 63.0 % of PHGPX activity retained respectively) for 48 hr prior to irradiation significantly increased the cell damage ($p < 0.01$), as measured by trypan blue assay (figure 4.26 c). These data suggest that TR alone may not be as crucial for protection of HaCaT against UVB-mediated damage as it is in protection against menadione-mediated cytotoxicity. The GPX's appear important for protection against UVB-mediated damage since inhibition of their activity by 100 μM GTG significantly increased the damage to HaCaT cells by UVB exposure. The decrease of cyGPX activity at higher concentrations of sodium selenite coinciding with loss of protection

against UVB-mediated damage further supports an important role for cyGPX in such protection. Only one experiment was performed to demonstrate increased susceptibility of HaCaT cells to UVB irradiation when pre-treated with 100 μM GTG. Ideally, further experiments should be carried out to confirm this in HaCaT cells, and also in primary keratinocytes.

Although Se has been demonstrated to modulate the cyGPX activity of HaCaT cells, no data is available on the cyGPX protein concentration. Investigation of whether the modulation of cyGPX concentration follows the pattern of the activity is needed to further elucidate the underlying mechanisms. Selective inhibition of the GPXs by means other than GTG (for example, antisense technology, or specific antibodies) in skin cells exposed to UVB would provide further information on the importance of these selenoenzymes in protection against damage mediated by UV-irradiation.

Menadione model versus UVB model

Menadione is a redox-cycling compound used widely as a model oxidative stress agent, which generates ROS in cells. In comparison, UVB is a more physiological oxidative stressor, which can injure cells by means of ROS-mediated cascades as well as by direct effect of the energy of UV irradiation. UV irradiation is well known to cause lipid peroxidation (Black *et al.*, 1997; Fuchs, 1998; Girotti, 2001; Yuen and Halliday, 1997), and some studies report menadione to cause lipid peroxidation also (Sorg *et al.*, 2002; Tzeng *et al.*, 1995). However, other reports suggest a cytotoxic mechanism of menadione that is independent of lipid peroxidation (Comporti, 1989; Thor *et al.*, 1982), since menadione is an inhibitor of the propagation reactions of lipid peroxidation (Wills, 1972). Cytotoxicity by menadione also involves rapid GSH depletion, ROS generation, protein thiol oxidation, and perturbations in Ca^{2+} homeostasis (Chen and Cederbaum, 1997; Cho *et al.*, 1997; Santini *et al.*, 1996; Thor *et al.*, 1982), and DNA damage (Halliwell and Gutteridge, 1999; Woods *et al.*, 1997). In addition, menadione is a substrate for TR1 (Arnér *et al.*, 1999; Gromer *et al.*, 1998; Luthman and Holmgren, 1982) which introduces bias towards TR as the protective enzyme in cells exposed to menadione. TR may reduce menadione before it can produce a large quantity of ROS, but the same would not be true for physiological stressors such as UVB, whose ROS have to be detoxified after production.

It is unclear how the differently mediated types of stress relate to the intensity of the other. Because of the discrete nature of UV light absorption by chromophores, the location of the primary photochemical target plays a considerable role in the development of the oxidative stress, as well as free iron. Thus, data obtained with 'dark' oxidative stresses, such as menadione, cannot easily be extrapolated to those produced by UV light. Different

antioxidant protection systems may be important against differing types of oxidative stress. In human keratinocytes ferritin is more important in protection against t-BuOOH-mediated oxidative stress than against photochemically-induced oxidative stress (Giordani *et al.*, 2000). This may imply that the induction of oxidative stress by the different toxic agents differs in its mechanism.

Menadione is not a substrate for mitochondrial TR, unlike cytosolic TR (TR1) (Rigobello *et al.*, 1998). By using menadione our studies may have 'weighted' the protection in favour of the cytosolic form of TR. The relative importance of the different TR isoforms in protection against oxidative stress is unknown at present. *In vivo*, a large proportion of ROS generation is from mitochondria, so mitochondrial TR is likely to be important in antioxidant defence. ROS are produced in the cytosol of cells exposed to UV, demonstrating a requirement for antioxidant protection in this location. The cytoplasmic and mitochondrial compartments of the cell are known targets of menadione (Santini *et al.*, 1996). Both the cytosolic and mitochondrial forms of TR are likely to be vital for protection of the cell from oxidative stress, but may vary according to the type of stressor.

The ability of Se to override the effects of GTG

Se-deficient HaCaT cells pre-incubated with 40 nM selenite for 48 hr were afforded significant protection ($p < 0.05$) from menadione-mediated cytotoxicity (figure 4.20), but cells pre-incubated with 10 μM GTG were significantly more susceptible ($p < 0.05$) to such damage. When HaCaT cells were treated with 40 nM selenite *in addition* to 10 μM GTG, the up-regulation of selenoenzyme expression by Se supplementation was sufficient to override the effects of the GTG in the cells, offering significant protection ($p < 0.05$) despite the loss of a percentage of the TR activity (> 80 %) and/or GPX activity. The level of protection provided to cells which had received selenite treatment alone or selenite in addition to GTG was almost identical. The corresponding selenoprotein activities for such pre-incubations were measured.

HaCaT cells pre-incubated with GTG (10 μM) alone had significantly lower TR ($p < 0.0001$) and cyGPX ($p < 0.05$) activities than controls (figure 4.21). Both TR activity and cyGPX activity were significantly increased ($p < 0.001$, and $p < 0.01$, respectively) by pre-incubation with selenite alone compared to controls. Pre-incubation with sodium selenite followed by GTG resulted in significantly augmented TR and cyGPX activities ($p < 0.05$, and $p < 0.01$ respectively) compared to those with GTG alone. However, the TR activity of HaCaT cells incubated with selenite followed by GTG was significantly lower ($p < 0.01$) than cells incubated with sodium selenite alone, whilst the cyGPX activity of these two treatment groups was not significantly different. The cyGPX activity was induced by selenite to a

greater extent, despite the GTG-mediated inhibition, than the TR activity. This may imply that Se upregulates the expression of more selenoprotein, which overcomes the protein already inhibited by GTG. The upregulation of cyGPX may compensate for the loss of TR activity, i.e. the protection observed in cells treated with selenite and GTG in conjunction may be largely due to cyGPX.

Inhibition of TR activity may lead to a number of potentially pro-oxidant effects in the cell, including either decreased TR activity, reduced Trx activity, decreased GSH concentration (and thus increased GSSG), increased Trx expression, increased expression of NFκB-dependent proteins, or increased expression of TR (Nordberg and Arnér, 2001). This further emphasises the inter-dependence of antioxidant systems of the cell.

Sub-cellular localisation of TR in skin cells

Immunohistochemical staining for Trx in human skin fibroblasts has revealed Trx to be located mainly in the cytoplasm and also around the nuclear membrane (Didier *et al.*, 2001). It would seem a reasonable assumption that TR must be localised to the same areas to keep Trx in its functional reduced state. TR can be detected in nuclei, mitochondria, lysosomes, microsomes, and cytosol (Chen *et al.*, 2002), and in the perimembraneous area of the plasma membrane (Hansson *et al.*, 1986; Rozell *et al.*, 1985) as well as the granular endoplasmic reticulum and cisternae of the Golgi body (Rozell *et al.*, 1988). The principal site of TR activity is the cytosol, where all TR isoforms are synthesized. Our immunocytochemical analysis of TR in skin cells showed TR to be localised largely in the cytosol and around the nuclear membrane for human primary keratinocytes and HaCaT cells. Dividing HaCaT cells appeared to have more intense staining for TR than did non-dividing cells. One function of Trx is as a growth factor, which is required to be in a reduced state to carry out this function. Rozell *et al.* suggested variations in immunoreactivity of TR in non-proliferating and differentiated cells to be related to their metabolic activity (Rozell *et al.*, 1985).

Schallreuter *et al.* have suggested that TR is located on the plasma membrane of keratinocytes. The immunohistochemistry presented here suggests that TR is largely cytoplasmic rather than membrane-associated. Although the studies of Rozell *et al.* showed TR to be localised to the perimembraneous area of the plasma membrane of salivary gland cells, secretory gland cells of the pancreas, and hepatocytes (Hansson *et al.*, 1986; Rozell *et al.*, 1985), this was not true for skin cells. Preliminary studies attempting to separate the subcellular fractions of [^{75}Se]-labelled skin cells during the studies presented here were unsuccessful. Such studies need to be repeated to provide further clarification of the specific localisation of TR in skin cells. Unsaturated membrane lipids are a crucial target for singlet

oxygen and ROS-mediated attack in photodynamic reactions, and modifications of such lipids may play a crucial role in UV-induced skin cancer, drug-sensitized phototoxicities, and anti-tumour PDT treatments (Girotti, 2001).

Comparison of inhibitors in the DTNB and insulin assay systems for TR

The DTNB and insulin assays for measurement of TR activity correlate well in their measurements of TR activity in calf liver and HeLa cells respectively (Zhong *et al.*, 1998) (Gorlatov and Stadtman, 1998). In these assay systems, the substrate is either DTNB, or, in the alternative assay, Trx (oxidised) and insulin (Gorlatov and Stadtman, 1998; Zhong *et al.*, 1998).

Various compounds were tested in the DTNB and insulin assay systems for TR activity in human placental cytosol. Comparing the two assay systems directly, a concentration of 1.11 mM azelaic acid, 0.28 mM anthralin, and 0.56 mM13-cis retinoic acid caused significantly different levels of inhibition between the two assay systems ($p < 0.001$; $p < 0.0001$; $p < 0.0001$ respectively). Significant inhibition was evident in the insulin assay, but not in the DTNB assay. Using azelaic acid, Becker *et al.* (2000) found no inhibition of human TR activity (Becker *et al.*, 2000). Kroll *et al.* (1999) found azelaic acid to have no inhibitory effect on spin label reduction by TR (Kroll *et al.*, 1999). Rigobello *et al.* (1998) found inhibition of mitochondrial TR activity by 100 μM and 500 μM 13-cis retinoic acid using the DTNB assay (Rigobello *et al.*, 1998). We only assessed the effects of inhibitors on the cytosolic form of TR however.

Schallreuter and Wood used their bioassay for TR, based on the decrease in electron spin resonance spectroscopy (ESR) amplitude of a cationic nitroxide spin label, a reduction proposed to be specific for TR in epidermis, to investigate TR inhibitors. Reduction of spin label on skin, on keratinocytes, melanocytes and purified *E. coli* TR was inhibited by thioprotein inhibitors, anthralin, azelaic acid and 13-cis retinoic acid. However these studies have been criticised because of the non-specific nature of the assay employed for measuring TR (Fuchs, 1988) (Fuchs *et al.*, 1990). Fuchs suggested that such inhibitors are not specific for TR. For example, azelaic acid inhibits tyrosinase and several oxidoreductases *in vitro* (Nazzaro-Porro, 1987); p-chloromercuribenzoate can inhibit many thiol mediated enzymatic and non-enzymatic reactions; anthralin is a strong reducing agent that may unspecifically modulate various enzymes including oxidoreductases (Fuchs *et al.*, 1990). In addition, nitroxides are reduced to ESR silent products by a variety of different NADH- or NADPH-dependent oxidoreductases in microsomes, mitochondria and plasma membranes (Fuchs, 1988).

Fuchs *et al.* also criticized the bioassay for TR regarding the fact that the nitroxide spin label did in fact penetrate the lipid bilayer of cell membranes, and propose the possibility that for different cell types, sites of intracellular reduction must be evaluated carefully. Schallreuter *et al.* had suggested that their radical substrate was selectively reduced by TR at the outer surface of the cell (Schallreuter and Wood, 1986). The studies presented in this thesis favour the opinion of Fuchs *et al.* in that the compounds suggested to be inhibitors of TR did not inhibit TR activity overall, as measured by the DTNB and insulin assay systems, and immunohistochemistry in skin cells did not reveal TR to be localised to membranes, but largely to the cytosol.

A further complication is that Schallreuter and Wood assessed the effect of inhibitor compounds on *E.Coli* TR using the DTNB assay with Trx addition for the majority of their studies. *E.Coli* TR requires Trx to reduce DTNB, but the mammalian enzyme does not. It is possible that these inhibitors were inhibiting Trx in the Schallreuter and Wood assay systems. Anthralin and 13-cis retinoic acid inhibited TR activity in human placental cytosol to a greater degree when measured by insulin assay than DTNB assay in the studies presented here. This further suggests the possibility of Trx inhibition since the insulin assay employs Trx in its linked assay system, whereas the DTNB assay system does not. Further studies should be carried out to either validate or invalidate these compounds as useful tools with which to study TR or Trx.

Conclusions

The HaCaT cell line is a suitable model for human keratinocytes in which to study selenoproteins and protection against oxidative stress. At sodium selenite concentrations required for optimal expression of TR, cyGPX and PHGPX, protection was afforded against damage mediated by menadione or UVB irradiation in HaCaT cells. This protection would appear to be due to the selenoprotein induction rather than a direct effect of sodium selenite since menadione was not directly detoxified by sodium selenite, and the cytotoxic damage mediated by menadione was exacerbated by the presence of selenite. Further evidence of the importance of the selenoproteins in protection against oxidative stress was provided by the observation that inhibition of TR activity alone in HaCaT cells increased their susceptibility to damage by menadione. This was not the case for damage by UVB, possibly indicating a differing role for TR against different types of oxidative stress, and that menadione and UVB differ in their cytotoxic mechanism.

At concentrations of sodium selenite higher than 100 nM, protection against UVB-mediated injury was lost in HaCaT cells. This was not the case with menadione cytotoxic insult, again highlighting the possibility of a differing cytotoxic mechanism, and protection mechanism. At

the sodium selenite concentrations at which protection was lost, cyGPX activity was seen to decrease. Such a decrease in activity was not seen with TR. This implies that the loss of protection may be due to the loss of cyGPX activity, and underlines the importance of cyGPX in Se-mediated protection against UVB damage. The underlying mechanism of the loss of cyGPX activity and loss of protection against UVB did not appear to be due to cytotoxicity of the sodium selenite. Negative feedback of the Se metabolite selenide or loss of GSH through GSSG formation may account for the loss of activity.

The dose of Se used for protection against damage by UVB is crucial. This has clinical implications for Se supplementation in community, i.e. supplementation with Se decreases risk for some cancers but may be ineffective against skin cancer, or even increase the risk.

From the work presented here, it is not certain that Se supplementation would decrease the incidence of skin cancer formation. However, Se supplementation *in vitro* does protect skin cells from damage induced by UVB irradiation. Sodium selenite induces selenoprotein expression at lower concentrations than selenomethionine. Selenite also provides protection from UVB-mediated damage at lower concentrations than selenomethionine (Rafferty, 2000). However, selenite is more toxic than selenomethionine, and doubts have been raised over whether it is the most suitable form for dietary Se supplementation (Schrauzer, 2001; Whanger, 2002). Se supplementation with selenomethionine may be preferable.

CHAPTER FIVE
THIOREDOXIN REDUCTASE AND CYTOPLASMIC GLUTATHIONE PEROXIDASE ACTIVITY IN HUMAN FOETAL AND NEONATAL LIVER

5.1 INTRODUCTION

The TR/Trx system has been implicated in a number of cellular processes including regulation of cell growth, apoptosis and the modification of the activity of transcription factors and receptors (Holmgren, 1989; Holmgren and Björnstedt, 1995). Mutant redox-inactive forms of Trx are incapable of stimulating cell growth or inhibiting apoptosis suggesting that Trx must be reduced to exert its effects on cell growth (Gallegos *et al.*, 1996). TR catalyses the NADPH-linked reduction of Trx and treatment of cells with the TR inhibitors doxorubicin or diaziquinone leads to an inhibition of ribonucleotide reductase activity, and inhibition of cell growth (Mau and Powis, 1992).

In addition to its growth promoting properties, TR also acts as an antioxidant either directly or through the action of Trx. TR can reduce and detoxify lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides directly using NADPH as a cofactor (Björnstedt *et al.*, 1995). In addition, TR is able to regenerate bioactivity in proteins inactivated by oxidative stress (Ejima *et al.*, 1999b; Spector *et al.*, 1988) and can also regenerate ascorbic acid from dehyroascorbate (May *et al.*, 1997).

Regulation of TR expression is through a number of factors including Se supply (Gallegos *et al.*, 1997), redox state of the cell (Sun *et al.*, 1999), oxidative stress (Ejima *et al.*, 1999a) and also through activation of protein kinase C (PKC) (Anema *et al.*, 1999). Increases in Se supply and oxidative stress lead to increased expression of TR (Ejima *et al.*, 1999a; Gallegos *et al.*, 1997), whilst activation of PKC may decrease (Anema *et al.*, 1999) or increase (Kumar and Holmgren, 1999) the expression of the enzyme. In the newborn primate lung oxygen appears to be an important factor in promoting an increased expression of the TR/Trx system (Das *et al.*, 1999). The process of childbirth is associated with an increase in oxidative stress in the lungs. With the first few postnatal breaths taken, the lung undergoes hyperoxia due to an abrupt change from the *in utero* PO_2 of 20 – 25 mm Hg (2.66 – 3.33 KPascal) to 100 mm Hg (13.3 KPascal) in air breathing (Kim *et al.*, 2001; Robles *et al.*, 2001). During hyperoxia there is increased generation of ROS including H_2O_2, which can react with Fe (II) to form the highly-toxic $OH^{\bullet}$.

The selenoenzyme cytoplasmic glutathione peroxidase (cyGPX) is also considered to exert powerful antioxidant function in the cell cytoplasm (Brigelius-Flohé, 1999), and the expression of cyGPX can be increased in situations of oxidative stress (Mitchell *et al.*, 1996) and when Se supply is increased (Brown *et al.*, 2000). The essential role of Se for mammalian development is illustrated by the finding that disruption of the mouse $tRNA^{Sec}$ gene results in early embryonic lethality (Bösl *et al.*, 1997).

The association between TR expression, cell growth and oxidative stress has lead us to speculate that changes in TR expression may be important in human foetal development.

The aims of the study in this chapter were:-

- to examine TR concentration and TR activity, and cyGPX activity, in human liver cytosol obtained from 7 foetuses (gestational age 16 to 20 weeks) and 5 neonates (aged 1 day to 15 weeks)
- to assess any correlations between TR and cyGPX in the hepatic cytosols

5.2 MATERIALS AND METHODS

5.2.1 Liver samples

Human liver tissue was obtained at autopsy from 7 foetuses (16-20 weeks gestation), and 6 term neonates who survived up to between 1 day and 15 weeks postnatally. Post-mortem time varied between 1 and 48 h after death. The study was approved by the Paediatric-Reproductive Medicine Ethics of Medical Research Sub-Committee of Lothian Health Board and the Ethics Committee of Tayside Health Board. Informed written consent was obtained from relatives prior to removing tissue.

5.2.2 Preparation of hepatic cytosols

Tissue was homogenised on ice, in 3 volumes of HEPES buffer (10 mM; pH 7.4) containing 2-mercaptoethanol (3 mM) and sucrose (0.25 M), using a glass Potter-Elvehjem homogeniser with a motor-driven Teflon pestle. The homogenate was centrifuged at 10,000 *x g* for 15 min, and the supernatant centrifuged at 100,000 *x g* for 1 hr. Aliquots of cytosol were snap-frozen on dry ice and stored at – 80°C.

Prior to assay for TR activity, cytosols were treated using Centricon-10 concentrator tubes (Amicon, MA, USA) to remove mercaptoethanol (which interferes with the TR activity assay). Cytosol (200 μl) was dispensed into the concentrator tube, together with 180 μl of assay buffer (100 mM potassium phosphate, 50 mM potassium chloride, 10 mM EDTA, 0.2 mg/ml BSA; pH 7.0). The concentrator tube was then centrifuged at 5,000 *x g* for 1 hr. After centrifugation, the filtrate was removed and a further 180 μl of buffer was added to the sample, and the centrifugation step repeated. At the end of the procedure, all samples were made up to a volume of 200 μl using assay buffer.

5.2.3 Measurement of cyGPX activity, TR activity and TR concentration

cyGPX and TR activity were measured as described in sections 2.3.7 and 2.3.5.1 respectively. All samples were measured in duplicate. Results were corrected for cytosol protein content, measured by the Bradford method (section 2.3.9). The TR concentration was measured in hepatic cytosols using the RIA detailed in section 2.3.6.

5.2.4 Statistical Analysis

The significance of the differences in TR concentration, TR activity and cyGPX activity between foetal and neonatal cytosols was assessed using the students 't' test with Welch correction for unpaired data.

5.3 RESULTS

5.3.1 Cytoplasmic glutathione peroxidase (cyGPX) activity , thioredoxin reductase (TR) activity and concentration in hepatic cytosols

The results are shown in Figures 5.01 and 5.02. The activity of TR was significantly greater ($p < 0.0005$) in foetal liver (median 2.05 U/g protein, inter-quartile range 1st to 3rd 1.76 - 2.47 U/g protein) than in the neonatal liver (0.65, 0.44 - 0.74 U/g protein). Similarly the concentration of TR in foetal liver (43.56, 37.92 - 50.80 μg/g protein) was significantly higher ($p < 0.05$) than the concentration found in neonatal liver (11.59, 8.70 - 14.99 μg/g protein).

The activity of cyGPX activity in the foetal cytosols (199.8 U/g protein ;143.9 - 227.9 U/g protein), was significantly greater ($p < 0.005$) than that found in the neonatal cytosols (77.0 U/g protein; 58.4 - 110.3). There were strong correlations between cyGPX activity and TR concentration ($r^2 = 0.58$; $p < 0.002$) and cyGPX activity and TR activity ($r^2 = 0.4$; $p < 0.02$) (Figure 5.03).

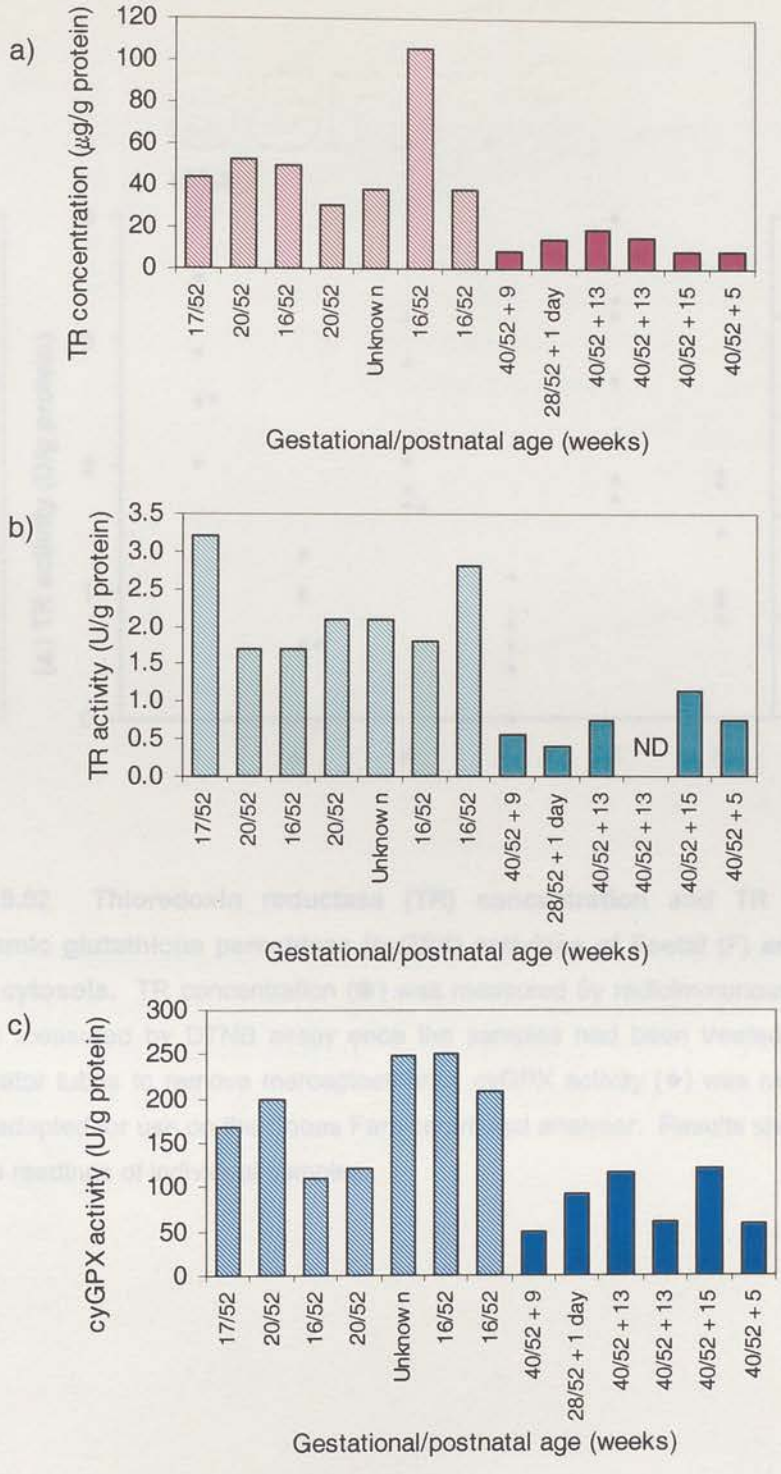


Figure 5.01 Thioredoxin reductase (TR) concentration (a) and TR activities (b), and cytoplasmic glutathione peroxidase (cyGPX) activities (c) of foetal (hatched bars) and neonatal (solid bars) hepatic cytosols. TR concentration was measured by radioimmunoassay; TR activity was measured by DTNB assay once the samples had been treated with Centricon-concentrator tubes to remove mercaptoethanol. Results shown are those of duplicate measurements of individual samples. ND = not determined.

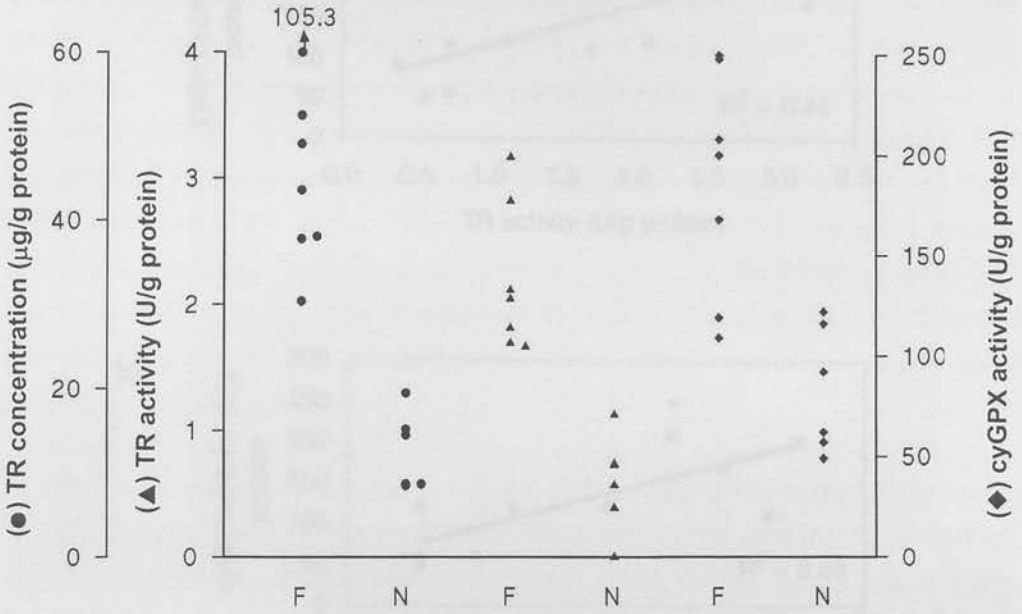


Figure 5.02 Thioredoxin reductase (TR) concentration and TR activities, and cytoplasmic glutathione peroxidase (cyGPX) activities of Foetal (F) and Neonatal (N) hepatic cytosols. TR concentration (●) was measured by radioimmunoassay; TR activity (▲) was measured by DTNB assay once the samples had been treated with Centricon-concentrator tubes to remove mercaptoethanol; cyGPX activity (◆) was measured using a method adapted for use on the Cobas Fara centrifugal analyser. Results shown are those of duplicate readings of individual samples.

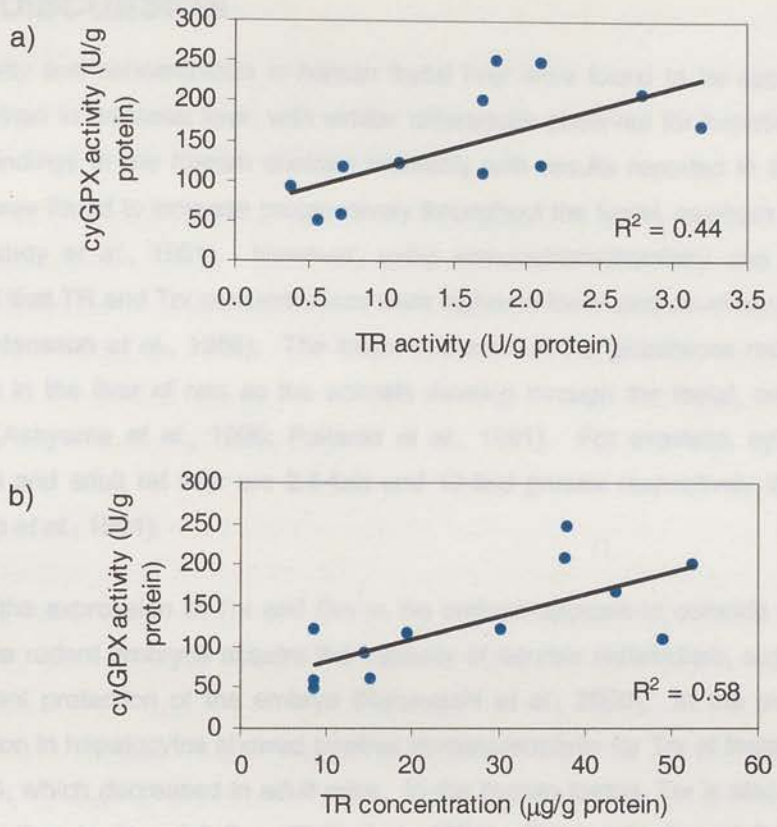


Figure 5.03 Correlation between thioredoxin reductase (TR) activity and cytoplasmic glutathione peroxidase (cyGPX) activity, and TR concentration and cyGPX activity in foetal and neonatal hepatic cytosols. The correlation of TR activity with cyGPX activity (a) gave an R^2 value of 0.44 ($p < 0.02$), and the correlation of TR concentration with cyGPX activity (b) an R^2 value of 0.58 ($p < 0.002$).

5.4 DISCUSSION

TR activity and concentration in human foetal liver were found to be approximately 3-fold greater than in neonatal liver, with similar differences observed for hepatic cyGPX activity. These findings in the human contrast markedly with results reported in the rat where TR activity was found to increase progressively throughout the foetal, newborn and adult stages (Demarquoy *et al.*, 1991). However, using immunohistochemistry one study in the rat reported that TR and Trx concentrations were higher in foetal and developing cells than adult tissue (Hansson *et al.*, 1986). The major enzymes of the glutathione redox cycle tend to increase in the liver of rats as the animals develop through the foetal, neonatal and adult stages (Asayama *et al.*, 1996; Pallardo *et al.*, 1991). For example, cyGPX activities in neonatal and adult rat liver are 2.4-fold and 13-fold greater respectively than in the foetus (Pallardo *et al.*, 1991).

In mice the expression of Trx and Grx in the embryo appears to coincide with the stage at which the rodent embryos acquire the capacity of aerobic metabolism, suggesting a role in antioxidant protection of the embryo (Kobayashi *et al.*, 2000). In the mouse foetus, Trx expression in hepatocytes showed positive immunoreactivity for Trx at foetal days 11.5, 13.5 and 16.5, which decreased in adult mice. In the human foetus, Trx is also expressed more intensely than in the adult liver (Fujii *et al.*, 1991). The localisation of Trx in the cell may reflect the functional state of the cell, or its phase in the cell cycle. In addition to a protective role against ROS, Trx and Grx may be involved in cell proliferation and differentiation in various tissues. Their roles may differ in varying tissues.

We can find no previous reports of TR ontogeny in humans but our results suggest that the rat model does not reflect the pattern observed in the human. Similar discrepancies have been found between rat and human for the ontogeny of other selenoenzymes in the liver, including type I and type III iodothyronine deiodinases (Richard *et al.*, 1998). Furthermore, our observations suggest that the changes in selenoenzyme expression seen around birth in the rat may not be due to maturation of selenoenzyme expression but rather a physiological regulation process that is not yet fully understood.

We found that cyGPX activity was approximately 3-fold higher in foetal than neonatal liver. Asikaninen *et al* (Asikainen *et al.*, 1998) have reported that cyGPX expression does not change significantly between the foetal and neonatal period in humans. The reasons for the discrepancy between our results and those of Asikaninen *et al.* are unclear, although their data for cyGPX activities showed a markedly skewed distribution.

Developmental changes in cyGPX expression have been observed in rat lungs where it increased after birth, especially when exposed to high oxygen tensions (Clerch and Massaro, 1993). Developmental changes in cyGPX have also been demonstrated in rat intestine (Tauchi *et al.*, 1991). Two weeks after birth, cyGPX protein became undetectable in duodenum and disappeared after weaning in ileum.

TR has many functions acting alone or in concert with thioredoxin. The TR/Trx system may modify cell growth (Gallegos *et al.*, 1996), exhibit oncoprotein-like properties (Koishi *et al.*, 1997) and promote cell proliferation by increasing cellular resistance to apoptosis (Gallegos *et al.*, 1996) (Baker *et al.*, 1997). The association between TR expression and cell growth might thus suggest that changes in TR expression may provide a mechanism by which foetal and neonatal development is controlled.

Alternatively, changes in TR expression in the foetus and neonate may be linked to oxidative stress, modified redox state of the cell or changes in calcium signalling or Se supply (Das *et al.*, 1999; Howie *et al.*, 1998). In baboon lung, TR is expressed constitutively at low levels in the foetus, and increases rapidly with the onset of O_2 or air breathing at birth (Das *et al.*, 1999). Similarly the induction of GPX expression is frequently observed in situations where there is an increased oxidant stress; for example, thyroidal cyGPX increases in iodine deficiency (Brown *et al.*, 2000). Oxygen also induces Mn-SOD, but not Cu/Zn-SOD, in neonatal rat lung (Stevens and Autor, 1977), and induces Prx I but not Prx II (Kim *et al.*, 2001). Prx I expression is also upregulated by oxygen in the newborn primate lung (Das *et al.*, 2001). The situation in the lung may be different to the other organs of the neonate in its antioxidant regulation since it is directly exposed to hyperoxic stress.

It has been suggested that changes in TR activity may be linked to the redox state of the cell, with a consequent effect on redox-regulated cell signalling (Sun *et al.*, 1999). These workers proposed that intracellular generation of ROS oxidises the selenol group of TR, with a consequent decrease in enzymic activity. The resulting oxidation of Trx would then modulate Trx-dependent cellular constituents, including transcription factors (e.g. NFκB) and antioxidant enzymes (e.g. thioredoxin peroxidase/peroxiredoxin). We have observed that TR activity and concentration change in parallel between foetal and neonatal liver suggesting that this mechanism does not explain the differences in TR activity between the foetus and neonate.

Whilst TR may act as a growth factor, our observations that activities of both TR and cyGPX are higher in the foetus than the neonate could be explained by induction of these antioxidant enzymes by oxidative stress in foetal liver. Indeed we found very strong correlations between cyGPX activity and TR activity and expression. Although it is possible

that the parallel changes in TR and cyGPX may have resulted from changes in Se supply after birth this is highly unlikely. One liver was obtained from a 1-day-old neonate and in this tissue, TR expression was lower than that found in any of the foetal livers studied. Moreover, changes of selenoprotein expression in response to Se supply take weeks rather than days to occur.

In conclusion, we have found that the activities of the antioxidant selenoenzymes TR and cyGPX are higher in the foetal than the neonatal liver. We speculate that these differences may reflect altered states of oxidative stress during development.

CHAPTER SIX CONCLUDING REMARKS

Atherosclerosis is the leading cause of mortality in the Western world and damage to the endothelium by reactive oxygen species (ROS) leads to endothelial dysfunction, favouring atherogenesis. Skin damage caused by ultraviolet (UV) irradiation involves ROS and such damage is thought to be involved in the pathogenesis of skin cancer.

The trace element selenium (Se) exerts many of its effects through modifying the expression of specific selenoproteins. The glutathione peroxidases (GPX) and thioredoxin reductases (TR) are families of selenoproteins that may have antioxidant functions and are found in most tissues including the endothelium and the skin. Se supplementation can protect against UVB-induced damage in skin cells (Leccia *et al.*, 1993; Moysan *et al.*, 1995; Rafferty *et al.*, 1998a; Rafferty *et al.*, 1998b; Richard *et al.*, 1990), and oxidative damage to endothelial cells (Miller *et al.*, 2001; Ochi *et al.*, 1992; Thomas *et al.*, 1993). Although the underlying mechanisms of protection have not been fully elucidated, it has been proposed that the GPXs and possibly TR are important mediators of such protection. The relative importance of the individual selenoproteins in a protective role has not been assessed so far in the literature to date.

In this thesis the role of TR and the GPXs in protection of endothelial cells and skin cells from oxidative damage was studied. The EAhy926 cell line and HaCaT cell line were used as models of human endothelial cells and keratinocytes, respectively, and their suitability as such models was assessed. The modification of the expression of TR and the GPXs through Se supply and treatment with gold thioglucose formed the main studies to assess selenoprotein-mediated protection. Both physiological (oxLDL and UVB) and non-physiological (t-BuOOH and menadione) oxidative stress agents were used to investigate protection in *in vitro* endothelial cells and skin cells respectively.

The work in this thesis has provided evidence to demonstrate the following:-

1. The [^{75}Se]-selenoprotein profile of EAhy926 cells and HUVEC was similar. The basal levels and the induction of the selenoproteins by Se supplementation was similar in EAhy926 to HUVEC, suggesting that the EAhy926 cell line is a good model in which to study expression and function of these selenoproteins. BAEC differed considerably from HUVEC and EAhy926 cells in their selenoprotein expression, suggesting that BAEC are an unsuitable model for human EC.

2. The ability of sodium selenite to protect against oxidative damage from t-BuOOH or oxidised LDL was demonstrated in EAhy926 cells, and is likely to be through the modification of selenoprotein expression rather than a direct antioxidant effect. The concentrations of sodium selenite which provided protection against cytotoxicity from t-BuOOH or oxLDL maximally induced TR and cyGPX in EAhy926 cells.

3. The gold compound gold thioglucose (GTG) was shown to modify selenoprotein activity, with TR being more sensitive to inhibition of activity than either cyGPX or PHGPX in cultured cells. The relative importance of TR, cyGPX and PHGPX in the protection of cells against oxidative stress was thus investigated.

4. In EAhy926 cells, significant inhibition of TR activity alone, but not the GPXs, (1 μM GTG) rendered the cells more susceptible to oxidative damage from t-BuOOH or oxidised LDL. These results suggest that TR is important in protection of endothelial cells from oxidative damage resulting from oxidised lipids. Cells treated with GTG at a concentration that inhibited the activity of both TR and the GPXs were more susceptible to t-BuOOH toxicity ($p < 0.05$) than cells treated with 1 μM GTG. These data suggest that under normal circumstances both TR and the GPXs are involved in the prevention of oxidative damage to human EC.

5. The HaCaT cell line had similar levels of TR activity to primary keratinocytes. HaCaT cells are afforded protection against oxidative stress by similar concentrations of Se to those in primary keratinocytes. These data suggest that the HaCaT cell line is a suitable model in which to study selenoprotein function of keratinocytes.

6. The ability of sodium selenite to protect against oxidative damage from menadione or UVB irradiation was demonstrated in HaCaT cells. This is also likely to be through modification of selenoprotein expression. The sodium selenite concentrations that maximally induced TR and cyGPX were those which provided protection against damage from menadione or UVB.

7. In HaCaT cells, inhibition of TR activity alone, using 10 μM GTG, rendered the cells more susceptible to oxidative damage from menadione, but not to damage from UVB irradiation. The results here suggest that TR is more important in protecting keratinocytes from menadione-mediated oxidative stress than UVB-mediated stress. These results also suggest that the mechanism of cytotoxicity due to menadione exposure is different to that caused by UVB irradiation; this may be due to the fact that menadione is a substrate for TR1. Menadione may not be a suitable model agent to use to represent damage caused by irradiation with UVB.

8. Using UVB as the toxic agent, loss of protection was seen with increasing concentrations of sodium selenite greater than 100 nM. Loss of protection was associated with a loss of cyGPX activity ($p < 0.05$), but not of TR or PHGPX expression. When menadione was used as the oxidative stressor, no loss of protection was demonstrated at the higher selenite concentrations. This further highlights a potentially different cytotoxic mechanism, and protection mechanism. The loss of cyGPX activity may be due to negative feedback of the Se metabolite selenide, or loss of GSH through GSSG formation.

9. Both TR and cyGPX may also have an antioxidant role in the developing foetus. TR activity and concentration, and cyGPX activity in human foetal liver were approximately 3-fold greater than in neonatal liver. These findings in the human contrast markedly with results reported in the rat where TR and cyGPX activities increase through the foetal, to the newborn and adult stages. These results show that the rat is not a representative model for studying the role of cyGPX and TR in human development. These differences in antioxidant enzymes may reflect altered states of oxidative stress during development.

In conclusion, the activity of the selenoproteins TR, cyGPX and PHGPX, which are present in endothelial cells and skin cells, can be modified by Se supplementation and GTG treatment. The GPXs and TR are important contributors to the antioxidant defence mechanisms of endothelial cells and skin cells, and may have a role in protecting against atherogenesis and skin carcinogenesis. The GPXs and TR may be differentially regulated in cells, as well as being of differing importance in different cell types, and in protection against different types of oxidative stress. These antioxidant enzymes may operate in different cellular compartments.

Further work leading on from the studies presented here could be to investigate which components of oxLDL are detoxified by a selenoenzyme-dependent mechanism.

Impaired endothelium-dependent vasodilation and increased oxidative stress is seen in GPX (-/-) knockout mice (Forgione *et al.*, 2002). Development of TR knockout mice would be useful to complement data already obtained from cyGPX knockout mice on the role of these selenoproteins in protection against endothelial dysfunction.

Further studies of interest in skin cells would be to establish the mechanism of loss of cyGPX at higher sodium selenite concentrations. Investigations of cyGPX protein may be useful in ascertaining the mechanism. Studies in mice would be valuable to determine whether the loss of cyGPX activity is a mechanism that also occurs *in vivo*. Although there

may be justification for intervention to increase the daily intake of Se in the UK, there should be caution since the dosage is crucial for the above reason.

If an improvement can be made on the sensitivity of the PHGPX assay, the role of this selenoenzyme in protection against oxidative stress in skin cells and endothelial cells could be studied. Antisense probes, specific antibodies, or specific inhibitors of selenoproteins could be used to further investigate the role of individual selenoproteins in protection against oxidative stress. In addition, cell lines could be established to over-express TR.

Work to investigate the subcellular localisation of TR is required to definitively confirm the location of TR in skin cells. Subcellular fractionation of [^{75}Se]-labelled primary keratinocytes and HaCaT cells would provide data to confirm this.

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Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell line EAhy926 from damage by lipid hydroperoxides

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Abstract

The human endothelial cell line EAhy926 was used to determine the importance of selenium in preventing oxidative damage induced by *tert*-butyl hydroperoxide (*tert*-BuOOH) or oxidised low density lipoprotein (LDLox). In cells grown in a low selenium medium, *tert*-BuOOH and LDLox killed cells in a dose-dependent manner. At 555 mg/l LDLox or 300 μM *tert*-BuOOH, >80% of cells were killed after 20 h. No significant cell kill was achieved by these agents if cells were pre-incubated for 48 h with 40 nM sodium selenite, a concentration that maximally induced the activities of cytoplasmic glutathione peroxidase (cyGPX; 5.1-fold), phospholipid hydroperoxide glutathione peroxidase (PHGPX;1.9-fold) and thioredoxin reductase (TR; 3.1-fold). Selenium-deficient cells pre-treated with 1 μM gold thioglucose (GTG) (a concentration that inhibited 25% of TR activity but had no inhibitory effect on cyGPX or PHGPX activity) were significantly ($P<0.05$) more susceptible to *tert*-BuOOH toxicity (LC_{50} 110 μM) than selenium-deficient cells (LC_{50} 175 μM). This was also the case for LDLox. In contrast, cells pre-treated with 40 nM selenite prior to exposure to GTG were significantly more resistant to damage from *tert*-BuOOH and LDLox than Se-deficient cells. Treatment with GTG or selenite had no significant effect on intracellular total glutathione concentrations. These results suggest that selenium supplementation, acting through induction of TR and GPX, has the potential to protect the human endothelium from oxidative damage.

Keywords: Selenium; Endothelial cell; Thioredoxin reductase; Glutathione peroxidase; Low density lipoprotein; Human; EAhy926

1. Introduction

Endothelial cells are continually exposed to a pro-oxidant environment in the vasculature, and to the possibility of damage by reactive oxygen species, hydrogen peroxide and lipid peroxides, etc. [1–3]. Oxidised low density lipoprotein (LDLox) is an important mediator of oxidative damage to the endothelium [4–6], and is directly cytotoxic to human endothelial and smooth muscle cells [7–11]. Lipid peroxidation of LDL by metal ions or by cells in vitro gives rise to a large variety of primary and secondary products from the lipid constituents. Some of these products react with the lysine groups of apoprotein B, resulting in recognition by the scavenger receptor [7]. Other products are cytotoxic [11].

Oxidative damage to the endothelium by LDLox may be one of the principal mechanisms in the pathogenesis of atherosclerosis [1–3]. Protection against oxidative damage is achieved through numerous enzymatic and non-enzymatic systems [12,13]. It has been suggested that the development and progression of atherosclerosis may be

Abbreviations: BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; EC, endothelial cells; FBS, foetal bovine serum; GSH, glutathione; GTG, gold thioglucose; cyGPX, cytoplasmic glutathione peroxidase; HCAEC, human coronary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; LDLox, oxidised low density lipoprotein; PHGPX, phospholipid hydroperoxide glutathione peroxidase; Se, selenium; *tert*-BuOOH, tertiary-butyl hydroperoxide; TR, thioredoxin reductase

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inhibited by selenium (Se). This essential trace metal exerts antioxidant actions through increased expression of selenoenzymes, such as the family of glutathione peroxidases (GPX) [14–17]. More recent evidence has suggested that the selenoenzyme thioredoxin reductase (TR; EC 1.6.4.5) may be particularly important in providing an antioxidant role in the human endothelium [17,18].

Studies using cultured animal endothelial cells have provided convincing evidence that Se is essential to provide maximum protection from oxidative damage. For example, bovine aortic endothelial cells (BAEC) in culture are more resistant to cytotoxic damage by photo-generated LDLox or *tert*-butyl hydroperoxide (*tert*-BuOOH), when pre-treated with sodium selenite compared with unsupplemented controls [16]. This resistance was ascribed to increased expression of cytoplasmic GPX (cyGPX; EC 1.11.1.9) and phospholipid hydroperoxide GPX (PHGPX; EC 1.11.1.12). The involvement of other selenoenzymes, such as TR, in such a protective role was not considered.

TR is a homodimeric selenoenzyme belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases, which include lipoamide dehydrogenase and glutathione reductase [19]. Each subunit of TR has a selenocysteine residue as the penultimate amino acid residue at the carboxyl terminal that is essential for catalytic activity of the enzyme [19]. Three isoforms of TR have been identified in humans, one mitochondrial and two cytoplasmic. They share considerable homology (reviewed in Ref. [20]). The predominant isoform of TR is the ubiquitous cytoplasmic form, TR1 [20]. TR is a multifunctional selenoprotein that, in conjunction with thioredoxin (Trx) and NADPH, forms a powerful dithiol-disulfide oxidoreductase system. TR1 can reduce and detoxify lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides directly using NADPH as a cofactor [19–21]. In addition, TR1 restores bioactivity in some enzymes inactivated by oxidative stress [22]. It can regenerate ascorbic acid from dehyroascorbate [23]. The TRs may also exert antioxidant action through their ability to reduce thioredoxin [19,20].

The GPXs have antioxidant function in the cell [24], with cyGPX catalysing the reduction of hydrogen peroxide and a variety of hydroperoxides [25]. PHGPX is capable of catalysing the reduction of phospholipid hydroperoxides and cholesterol hydroperoxides, many of which are not substrates for cyGPX [26]. TR can detoxify hydrogen peroxide and lipid peroxides more efficiently than the GPXs under certain conditions [21]. This, together with the high expression of TR in human EC [17,18], suggests that the selenoenzyme may be more efficient than the GPXs in defence against oxidative damage. Thus, it is possible that decreased expression and activity of these selenoproteins as a result of Se deficiency in man, increases the susceptibility of the endothelium to oxidative damage by LDLox or other lipid hydroperoxides.

Gold thioglucose (GTG) interacts with Se residues in selenoenzymes [27], and thus inhibits their activity. Selenoenzymes show marked variation in their sensitivity to inhibition by GTG. The GPXs are relatively resistant to inhibition by gold compounds. TR is very sensitive, having an IC_{50} ~ 1000-fold lower than that of the GPXs [28]. Gold compounds can be administered to animals in specific doses that can inhibit TR activity without modifying the activity of the GPX [29]. Thus, the use of different concentrations of GTG may offer a convenient tool to elucidate the relative importance of selenoenzymes in antioxidant defence of endothelial cells.

Sodium selenite pre-treatment affords protection against the harmful effects of *tert*-BuOOH in human endothelial cells from different sources [17]. However, no studies in human endothelial cells have investigated the possibility that Se can protect such cells from the harmful effects of LDLox. The human endothelial cell line EAhy926, established by hybridising primary human umbilical vein endothelial cells (HUVEC) with A549 human lung tumour cells [30], has been used in a number of functional studies. EAhy926 retain many of the differentiated functions common to primary endothelial cells beyond 100 passages. These functions include the expression of von Willebrand Factor [30], prostacyclin formation [31], and expression of endothelin-1 [32]. This cell line is susceptible to damage from LDLox [10]. We have used this as a model system to determine if human endothelial cells can be protected from the harmful effects of LDLox by selenite. In addition, we have used GTG at selective concentrations that predominantly inhibit TR, to examine whether the enzyme plays a significant antioxidant role in human endothelial cells.

2. Materials and methods

2.1. Chemicals and cell culture reagents

GTG, sodium selenite, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), hydrogen peroxide, hypoxanthine, glutathione, aminopterin, thymidine media supplement (HAT), lactate dehydrogenase (LDH) assay kit, NADPH, and *tert*-BuOOH were supplied by Sigma Aldrich, Poole, Dorset, UK.

Dulbecco's modified Eagle's medium (DMEM) with (25 mM HEPES) and 4500 mg/l glucose, phosphate-buffered saline (PBS), Earle's balanced salt solution (EBSS), foetal bovine serum (FBS) were supplied by Gibco, Life Technologies, Paisley, UK.

Cell culture plastics were supplied by Iwaki, Japan.

2.2. Cell culture

The human endothelial cell line EAhy926, derived from umbilical vein endothelial cells, was a kind donation from Professor C.-J.S. Edgell, University of North Carolina, Chapel Hill, NC, USA. The cell line was maintained in high glucose (4.5 g/l) DMEM containing 10% FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, and 0.8 mM thymi-

dine, in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. The cells were passaged weekly using 0.25% trypsin–0.02% EDTA solution. EAhy926 cells displayed the characteristic morphology of endothelial cells in culture and stained positively for von Willebrand Factor.

The selenium content of the basal medium (without FBS), determined by acid digestion followed by fluorimetric analysis [33,34], was 0.35 nM and was classified as 'selenium-deficient medium'.

2.3. Determination of cellular integrity by measurement of LDH retention

Cell viability was assessed, in 24-well plates, as the percentage retention of LDH by the cell layer after 20 h exposure to *tert*-BuOOH or LDLox. Intracellular LDH activity in cells and in the culture medium was determined using a kit method (Sigma Diagnostics), modified for use on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The culture medium (1 ml) was removed for analysis, and the cells washed twice with 1 ml EBSS. The cells were then lysed in 0.5 ml of 0.5% Triton X-100 (in PBS, pH 7.4). After 15 min, the cell lysates were collected and the wells were washed with a further 0.5 ml EBSS and the washings combined with the respective lysates. Cell debris in the culture medium and cell lysates was removed by centrifugation at 11,500 × *g* for 10 min prior to assay.

The LDH activity was also measured in culture media that had not been in contact with cells as a measure of endogenous LDH in the culture medium (blank). All results were blank-corrected. Results were expressed as percent LDH activity retained.

2.4. Total protein measurement

The Bradford assay [35] was used to measure total protein in the native LDL, oxLDL and cells using BSA as the standard. All enzyme activity results were corrected for total protein measured using this method.

2.5. Measurement of TR activity

TR activity was measured by an optimised method based on the method of Hill et al. [36] using DTNB (5 mM) as substrate in the presence and absence of 720 nM GTG (final concentration). This method was adapted for use on the Cobas FARA centrifugal analyser (Roche Diagnostics). All samples were measured in duplicate. Results were corrected for total protein content and one unit of TR activity was defined as 1 μmol of DTNB reduced per minute.

2.6. Measurement of cytoplasmic GPX, phospholipid GPX and total glutathione

cyGPX activity was measured by monitoring the rate of oxidation of NADPH at 340 nm in the presence of H_2O_2 (2.2 mM), using a Unicam UV/Vis spectrometer (UV4) linked to a computer installed with 'Vision' software [37]. All samples were measured in triplicate and results were corrected for total protein concentration. PHGPX activity was determined using the same assay system as for cyGPX, but with phosphatidyl choline hydroperoxide as the substrate. Total glutathione was measured according to Tietz [38], and adapted for use on a Cobas FARA centrifugal analyser. All samples were measured in triplicate and results corrected for total protein [35]. One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.

2.7. Preparation of LDLox

LDL was isolated from 300 to 450 ml of human citrate plasma (Blood Transfusion Service, Edinburgh) by ultracentrifugation using a Ti 45 rotor in a Beckman L8.55 ultracentrifuge (Beckman, Glenrothes, UK). Plasma (45 ml) was overlayered with 15 ml of a buffered saline solution (ρ 1.019 g/ml) containing EDTA (10 mg/l) and centrifuged for 18 h at 186,000 × *g* max at 4 °C. The very low density lipoproteins were discarded. The density of the infranatant (40 ml) was adjusted to 1.063 g/ml by addition of 14.7 ml of buffered saline (ρ 1.182 g/ml), overlayered with 5.3 ml of saline solution (ρ 1.063 g/ml) and recentrifuged for 18 h at 186,000 × *g*. The LDL fraction was removed and transferred into 30 cm dialysis tubing (7.5 mm diameter, Spectra/Por, MW cut-off 300,000; Medicel, London) and dialysed overnight against 5 l of PBS (pH 7.4, 0.2 g/l chelex resin). The combined dialysates of known protein concentration were divided, one part was kept as native LDL (control for experiments) and the other was used to prepare LDLox as follows. Approximately 25 ml native LDL was oxidised at 37 °C using $CuCl_2$ (Cu–protein ratio 0.16 μmol/mg protein), and the formation of conjugated dienes monitored at 234 nm. At peak absorbance (usually 60–90 min after initiation), 10% excess EDTA was added to stop lipid peroxidation and the volume of the lipoprotein fraction was reduced to ~ 5–10 ml using a 30,000 MW cut-off polyethersulfone filter and an Amicon 52 filtration unit (Amicon Millipore, Bedford, MA, USA). Traces of Cu^{2+} were then removed by chromatography over a Sephadex G25 column (PD-10; Amersham Pharmacia Bio-Technics, Uppsala, Sweden) using PBS as the eluant. The tube containing LDLox was flushed with a 0.22 μm filtered stream of argon, and the LDL stored at 4 °C until required (within 2–3 weeks). Native LDL was treated in an identical manner (filtration, chromatography, storage under argon) except the fraction was not exposed to Cu^{2+}.

2.8. Statistical analysis

Comparison of all data was performed using ANOVA and the Student's *t*-test (with Welch correction as appropriate) for unpaired data. Enzyme activities in Table 1 were

Table 1
The effect of sodium selenite supplementation on the activity of TR, cyGPX, and PHGPX in EAhy926 cells [mean (U/g protein) ± S.E.]

Selenite (nM)	TR activity (U/g protein)	cyGPX activity (U/g protein)	PHGPX activity (U/g protein)
0	2.0 ± 0.5	27.3 ± 1.6	5.7 ± 1.2
1	3.2 ± 0.1†	40.8 ± 3.9†	5.4 ± 0.8
10	5.3 ± 2.6*	115.4 ± 10.2‡	8.8 ± 2.0†
50	6.1 ± 0.4‡	138.5 ± 5.9‡	11.1 ± 1.1‡
100	5.1 ± 0.3*	182.5 ± 26.9‡	10.5 ± 2.5‡
200	4.7 ± 0.3*	166.2 ± 29.4‡	10.5 ± 1.1‡
1000	7.4 ± 1.1‡	153.7 ± 13.6‡	12.6 ± 0.7‡

Cells were cultured in 75-cm^2 flasks with selenite for 48 h. All measurements are for triplicate flasks, except the controls for which $n=4$. One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.

One unit of TR activity was defined as 1 μmol of DTNB reduced per minute.

*$P<0.01$ cf. control cells (ANOVA with Fisher's test for least significant difference).

†$P<0.05$ cf. control cells (ANOVA with Fisher's test for least significant difference).

‡$P<0.001$ cf. control cells (ANOVA with Fisher's test for least significant difference).

compared using ANOVA with Fisher's test for least significant difference.

3. Protocols

3.1. The ability of selenite to prevent tert-BuOOH and LDLox toxicity

EAhy926 cells were passaged into 24-well plates at a density of 5×10^5 cells/cm^2 and left to grow in high glucose (4.5 g/l) DMEM containing 10% FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, and 0.8 mM thymidine for 48 h.

To assess the optimal concentration of sodium selenite for protection of EAhy926 cells from *tert*-BuOOH-mediated cytotoxicity, cells were pre-incubated with a range of selenite concentrations (0–1000 nM) for 48 h before being washed twice with 1 ml of EBSS. For each experiment, a concentration of *tert*-BuOOH was found that would kill approximately 90% of Se-deficient cells and this was determined from a pilot experiment that was performed while the cells to be used for the main experiment were being preincubated with selenite. This concentration varied slightly between experiments ranging from approximately 200 to 300 μM *tert*-BuOOH. Cell damage was produced by incubating cells with *tert*-BuOOH for 20 h. LDH retention was measured as described above, all determinations being carried out in triplicate wells.

The ability of EAhy926 cells to resist LDLox toxicity in the presence or absence of 40 nM selenite was determined as for *tert*-BUOOH above. Medium containing either native or oxidised LDL (prepared from the same blood donation, and diluted to a common protein value in medium) was added to the cells at the specified concentrations and cells left to incubate for 20 h. LDH activity was then measured in the medium and cell lysates as described above, and percentage LDH retention calculated. All determinations were carried out in triplicate wells.

3.2. Induction of TR, cyGPX, and PHGPX activities in EAhy926 cells by selenite

EAhy926 cells were passaged into 75-cm^2 flasks and grown to 70% confluence. The cells then received medium containing 0, 1, 10, 40, 50, 100, 200 or 1000 nM sodium selenite (triplicate flasks for each selenite concentration, and quadruplicate flasks for the control) for 48 h. Following this incubation, the cells were washed twice with 10 ml EBSS, and harvested by scraping into 20 ml EBSS. Efficiency of harvesting was determined by light microscopy. The cells were then pelleted by centrifugation at $500\times g$ for 10 min. The EBSS was aspirated, and the pellets frozen at −70 °C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed and lysed by sonication (three pulses of 10 s using a Soniprep 150) on ice in 0.125 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1% Triton X-100 (peroxide- and carbonyl-free). The activities of the selenoenzymes were then determined as detailed above.

3.3. Optimisation of the GTG concentration

EAhy926 cells were passaged into 75-cm^2 flasks and grown to 70% confluence. The cells then received medium containing 0, 1, 10, or 100 μM GTG (triplicate flasks for each GTG concentration, and quadruplicate flasks for the control) for 48 h. Following the incubation, the cells were washed twice with 10 ml EBSS, and harvested via scraping into 20 ml EBSS. Efficiency of harvesting was checked by light microscopy. The cells were then pelleted by centrifugation at $500\times g$ for 10 min. The EBSS was aspirated, and the pellets frozen at −70 °C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed, lysed and the activities of the selenoenzymes were then determined as described above.

3.4. tert-BuOOH and LDLox toxicity in the presence of GTG

EAhy926 cells were passaged into 24-well plates in either normal medium or medium containing 40 nM selenite, and incubated for 48 h. After this time, the cells were washed twice with 1 ml EBSS. Cells then received normal unsupplemented medium, or the same medium containing either 1 or 10 μM GTG for 48 h. When the incubation was

finished, the cells were again washed, and fresh medium containing *tert*-BuOOH (0–250 μM) added for a 20h incubation. LDH retention was then measured as described above. All determinations were carried out in triplicate wells.

The effect of 1μM GTG on modifying the susceptibility of EAhy926 cells to LDLox was carried out in an identical manner to that described above using an LDLox protein concentration of 220 mg/l.

4. Results

4.1. Effect of selenite on tert-BuOOH and LDLox-induced cell damage

Pre-incubation of EAhy926 cells with sodium selenite for 48 h showed that selenite provided optimal protection from the cytotoxic effects of 300 μM *tert*-BuOOH at concentrations ranging from 10 to 50 nM ($P<0.0005$, Fig. 1). Selenite when added at a concentration of 1000 nM was significantly toxic to EAhy926 cells in the absence of *tert*-BuOOH ($P<0.05$).

There was a concentration-dependent cytotoxicity of LDLox (Fig. 2). Native LDL was not cytotoxic to EAhy926 cells at any of the concentrations tested. In the presence of 555 mg/l LDLox, only 19.8 ± 1.8% of cells survived in the absence of selenite. In contrast, 92.7 ± 0.4% of cells pre-incubated with 40 nM selenite for 48 h survived when exposed to (555 mg/l) LDLox ($P<0.0005$). This concentration of 40 nM selenite was chosen as this was the lowest concentration of selenite that gave optimal protection from *tert*-BuOOH (Fig. 1).

4.2. Effect of selenite on GPX and TR activity

Incubation of EAhy926 cells with 50 nM sodium selenite resulted in maximal expression of TR and PHGPX while maximal expression of cyGPX was achieved at a selenite concentration of 100 nM. Significant induction of cyGPX ($P<0.05$), and TR ($P<0.01$) was first achieved with a concentration of 1 nM selenite, and for PHGPX ($P<0.05$) with 10 nM selenite (Table 1).

4.3. Effect of GTG on GPX and TR activity

At a concentration of 1 μM GTG, 75 ± 7.0% of TR activity was retained ($P<0.05$ cf. control cells). There was no significant loss of cyGPX or PHGPX activity (Table 2). Using 10 μM GTG, 15 ± 10% of TR activity, 40 ± 4% of cyGPX, and 65 ± 3% of PHGPX activity were retained compared to control cells ($P<0.01$, $P<0.05$, and $P<0.005$, respectively). When GTG was added at a concentration of 100 μM, marked inhibition of all selenoenzymes was observed such that 0.5 ± 0.5%, 15.0 ± 1.6%, and 54 ± 7% of enzyme activities were retained for TR, cyGPX and PHGPX, respectively ($P<0.0005$, <0.0005, and <0.01 cf. control cells).

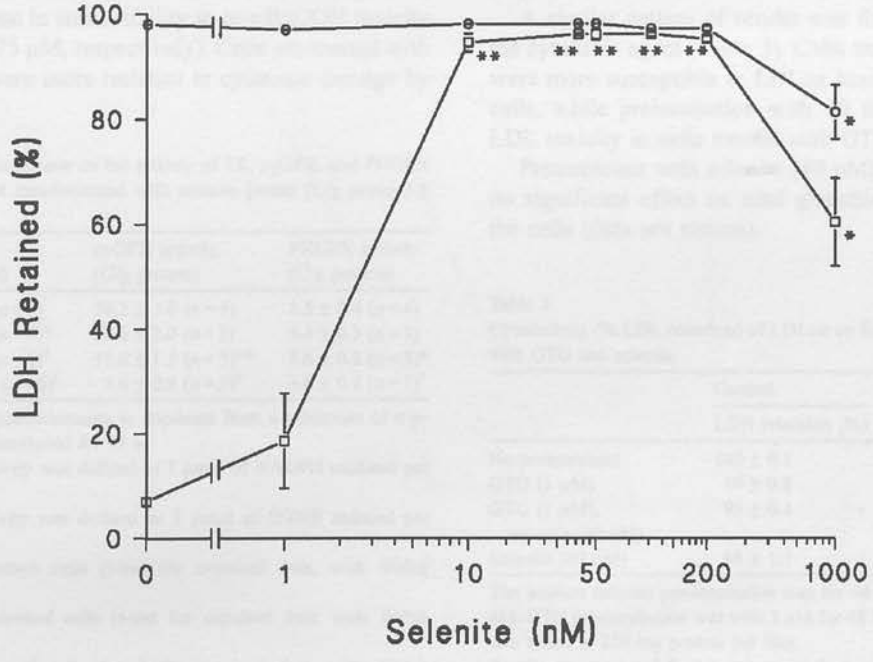

Fig. 1. Cytoprotection of EAhy926 cells from *tert*-BuOOH by sodium selenite pre-incubation. Pre-incubations with sodium selenite were for 48 h, prior to exposure to 0 μM *tert*-BuOOH (○) or 300 μM *tert*-BuOOH (□) for 20 h. Cytotoxicity was determined by LDL retention (%). All determinations are mean ± S.E. for triplicate wells. *$P<0.05$; **$P<0.0005$ cf. control cells (*t*-test for unpaired data, with Welch correction).

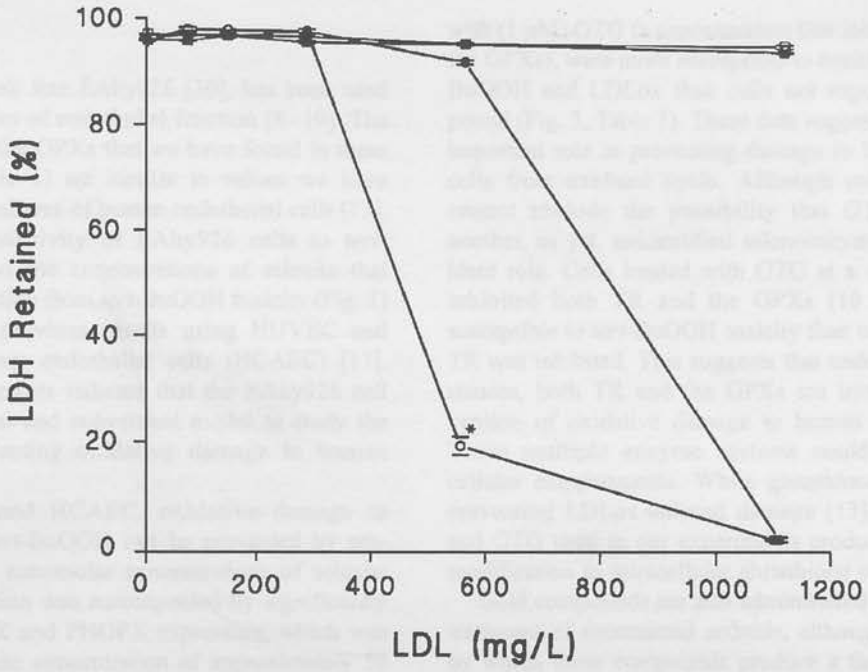

Fig. 2. Cytoprotection of EAhy926 cells from LDLox by preincubation with (40 nM) selenite. Cells were pre-incubated with sodium selenite (40 nM) for 48 h. Exposure of selenium-supplemented cells (closed symbols) or selenium-deficient cells (open symbols) to native LDL (squares) or LDLox (circles) was for 24 h. Cytotoxicity was determined by LDH retention. Results shown are means of triplicate wells ± S.E. *$P<0.0005$ cf. LDLox, cells plus Se (*t*-test for unpaired data).

4.4. Effect of GTG and/or selenite on oxidative cell damage

The LC_{50} for *tert*-BuOOH for Se-deficient cells was 175 μM. The same cells treated with either 1 or 10 μM GTG showed an increase in susceptibility to *tert*-BuOOH toxicity (LC_{50} = 110 and 75 μM, respectively). Cells pre-treated with 40 nM selenite were more resistant to cytotoxic damage by *tert*-BuOOH in the presence of 1 μM GTG than the corresponding cells pre-treated with 1 μM GTG alone (LC_{50} 195 cf. 110 μM $P<0.05$), or Se-deficient cells (Fig.3).

A similar pattern of results was found using LDLox as the cytotoxic agent (Table 3). Cells treated with 1 μM GTG were more susceptible to LDLox toxicity than Se-deficient cells, while preincubation with 40 nM selenite prevented LDL toxicity in cells treated with GTG.

Pretreatment with selenite (40 nM) or GTG (1 μM) had no significant effect on total glutathione concentrations in the cells (data not shown).

Table 2
The effect of gold thioglucose on the activity of TR, cyGPX, and PHGPX in EAhy926 cells not supplemented with selenite [mean (U/g protein) ± S.E.]

GTG (μM)	TR activity (U/g protein)	cyGPX activity (U/g protein)	PHGPX activity (U/g protein)
0	1.6 ± 0.1 (n=7)	39.2 ± 3.0 (n=4)	5.5 ± 0.4 (n=4)
1	1.2 ± 0.1 (n=6)*	39.6 ± 2.0 (n=3)	5.4 ± 0.3 (n=3)
10	0.2 ± 0.1 (n=6)‡	15.6 ± 1.5 (n=3)**	3.6 ± 0.2 (n=3)*
100	0.01 ± 0.01 (n=6)‡	5.9 ± 0.6 (n=3)‡	3.0 ± 0.4 (n=3)†

Results are activity determinations in duplicate from a minimum of triplicate 75-cm^2 flasks incubated for 48 h.
One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.
One unit of TR activity was defined as 1 μmol of DTNB reduced per minute.
*$P<0.05$ cf. control cells (*t*-test for unpaired data, with Welch correction).
**$P<0.005$ cf. control cells (*t*-test for unpaired data, with Welch correction).
†$P<0.01$ cf. control cells (*t*-test for unpaired data, with Welch correction).
‡$P<0.0005$ cf. control cells (*t*-test for unpaired data, with Welch correction).

Table 3
Cytotoxicity (% LDL retention) of LDLox on EAhy926 cells pre-incubated with GTG and selenite

	Control	LDLox (220 mg/l)
	LDH retention (%)	LDH retention (%)
No pretreatment	100 ± 0.1	70 ± 2.5*
GTG (1 μM)	99 ± 0.8	60 ± 5.1*
GTG (1 μM), selenite (40 nM)	99 ± 0.4	99 ± 1.0
Selenite (40 nM)	98 ± 1.7	99 ± 1.6

The sodium selenite pre-incubation was for 48 h at a concentration of 40 nM. GTG pre-incubation was with 1 μM for 48 h. The cytoxicity of LDLox was tested at 220 mg protein per liter.
Results are mean ± S.E. determinations from triplicate wells.
Cells treated with GTG alone had significantly ($P<0.05$) more cell death than cells not treated with GTG or selenite.
* Significant ($P<0.05$) differences from control cells.

5. Discussion

The endothelial cell line EAhy926 [30], has been used for a number of studies of endothelial function [8–10]. The activities of TR and the GPXs that we have found in these EAhy926 cells (Table 1) are similar to values we have reported in primary cultures of human endothelial cells [17]. Furthermore, the sensitivity of EAhy926 cells to *tert*-BuOOH (Fig. 1) and the concentrations of selenite that confer optimal protection from *tert*-BuOOH toxicity (Fig. 1) are also similar to previous results using HUVEC and human coronary artery endothelial cells (HCAEC) [17]. These data taken together indicate that the EAhy926 cell line provides an ideal and convenient model to study the role of Se in preventing oxidative damage to human endothelial cells.

As in HUVEC and HCAEC, oxidative damage to EAhy926 cells by *tert*-BuOOH can be prevented by pre-incubation with low nanomolar concentrations of selenite (Fig. 1) [17]. Protection was accompanied by significantly increased TR, cyGPX and PHGPX expression, which was optimal with a selenite concentration of approximately 50 nM (Table 1). While *tert*-BuOOH is widely used as a model agent to induce oxidative stress, in vitro, LDLox is considered to be the principal agent that damages the endothelium and promotes atherogenesis. LDLox has also been shown to damage EAhy926 cells in culture [8]. Supplementation of cells with 40 nM selenite provides protection from oxidative damage initiated by LDLox (Fig. 2). Cells preincubated with (1 μM) GTG (a concentration that inhibited TR, but not the GPXs), were more susceptible to toxicity from both *tert*-BuOOH and LDLox than cells not exposed to this compound (Fig. 3, Table 3). These data suggest that TR plays an important role in preventing damage to human endothelial cells from oxidised lipids. Although unlikely, these data cannot exclude the possibility that GTG has inhibited another, as yet, unidentified selenoenzyme with an antioxidant role. Cells treated with GTG at a concentration that inhibited both TR and the GPXs (10 μM) were more susceptible to *tert*-BuOOH toxicity than cells in which only TR was inhibited. This suggests that under normal circumstances, both TR and the GPXs are involved in the prevention of oxidative damage to human endothelial cells. These multiple enzyme systems could act in different cellular compartments. While glutathione is important in preventing LDLox-induced damage [13], the doses of Se and GTG used in our experiments produced no significant modification to intracellular glutathione concentrations.

Gold compounds are also administered to humans for the treatment of rheumatoid arthritis, although the mechanism by which these compounds produce a therapeutic effect is unknown. Reglinski et al. [39] have shown that such treatment increases 'oxidative stress'. Our data shows that GTG increases the susceptibility of Se-deficient endothelial cells to oxidative damage and that this damage may be prevented by pre-treatment with selenite at doses that maximally induce the expression of the GPXs and TR (Fig. 3, Table 3). Countries, such as the United Kingdom, have a Se intake

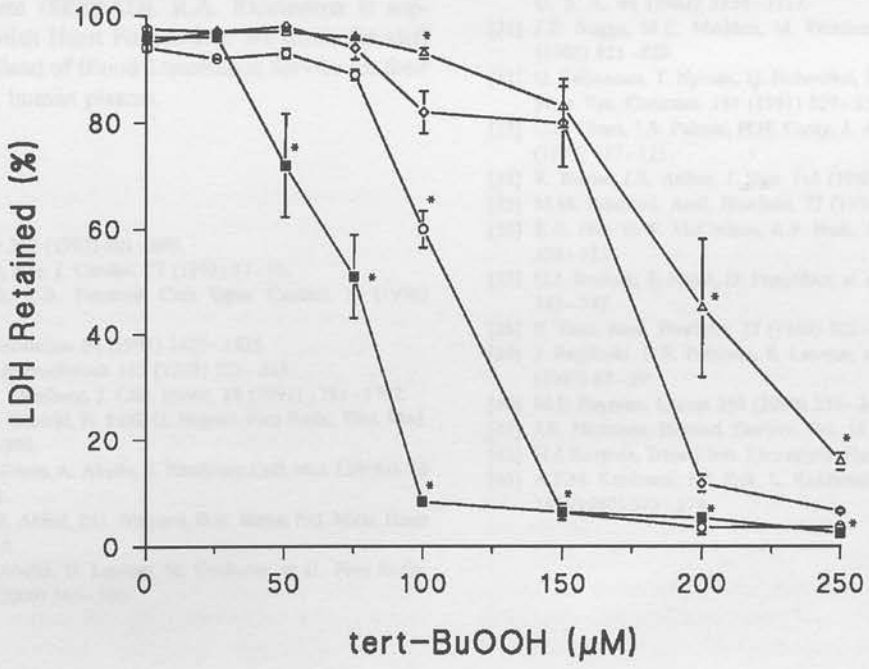

Fig. 3. Cytotoxicity profile of *tert*-BuOOH to EAhy926 cells pre-incubated with various combinations of GTG and selenite. (△) Selenite (40 nM) GTG (1 μM), (◇) No additions, (○) GTG (1 μM), (■) GTG (10 μM). Sodium selenite pre-incubation was for 48 h at a concentration of 40 nM. GTG pre-incubations were for 48 h. Results are mean ± S.E. determinations from triplicate wells. Significant ($P<0.05$) differences from control cells are shown by (*).

that is insufficient to maximally induce selenoenzyme expression [40]. It could thus be argued that in such populations, GTG treatment may lead to damage to the endothelium. If this were the case, such patients may benefit from selenium supplementation prior to treatment with GTG. However, if the efficacious effects of GTG on rheumatoid arthritis required the production of oxidative stress, it is possible that Se supplementation may prevent the therapeutic action of GTG. Clinical trials would be required to determine the potential benefits of Se supplementation prior to GTG treatment.

Finally, our observations support the view that a low Se status would promote endothelial injury and atherosclerosis initiated by LDLox in humans [41–43]. There are no reports to our knowledge that show in vivo how the Se content of the endothelium responds to Se supplementation. However, our results suggest that selenium supplementation at doses which optimise the expression of the TR and the GPXs may have significant beneficial effects when applied to populations that have an Se intake below that currently recommended. Properly conducted controlled trials of selenium supplementation are urgently required.

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Thioredoxin reductase and cytoplasmic glutathione peroxidase activity in human foetal and neonatal liver

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Abstract

Cytosolic thioredoxin reductase (TR) is an FAD-containing homodimeric selenoenzyme which, together with thioredoxin (Trx) and NADPH, forms a powerful oxidoreductase system. Cytoplasmic glutathione peroxidase (GPX-1) is a selenoprotein with antioxidant activity. The TR/Trx system has been associated with cellular processes including regulation of cell growth, and modification of activity of transcription factors. TR may also act as an antioxidant. We have measured TR activity, TR concentration, and GPX-1 activity in human hepatic cytosols from foetuses and neonates. The concentration of TR was significantly greater ($P < 0.05$) in foetal (43.6, 37.9–50.8 μg/g protein, median, interquartile range) than in neonatal liver (11.6, 8.70–15.0 μg/g). This was also true of TR activity which was 2.1, 1.8–2.5 U/g protein in foetal, and 0.65, 0.44–0.74 U/g protein in neonatal liver ($P < 0.0005$). Similarly, GPX-1 activity was significantly higher ($P < 0.005$) in the foetal (199.7, 144.0–227.9 U/g protein) than in neonatal (77.0, 58.4–110.3 U/g protein) hepatic cytosol. Overall, foetal liver expressed approx. 3-fold higher activities of TR and GPX-1 than neonatal liver.

Keywords: Hepatic; Thioredoxin reductase; Antioxidant; Fetal; Neonatal; Measurement

1. Introduction

Thioredoxin reductase (TR) is a homodimeric selenoenzyme belonging to the flavoprotein family of pyridine nucleotide-disulphide oxidoreductases that include lipoamide dehydrogenase and glutathione reductase. Each subunit of TR has a selenocysteine residue as the penultimate amino acid residue at the carboxyl terminus that is essential for catalytic activity of the enzyme [1]. Three isoforms of TR have been identified in humans, one mitochondrial [2] and two cytoplasmic [3], and they share considerable homology. The predominant isoform of TR quantitatively is the ubiquitous cytoplasmic form, TR1 [3].

TR is a multifunctional selenoprotein that, with thioredoxin (Trx) as a substrate and NADPH as a cofactor, forms a powerful dithiol-disulphide oxidoreductase system. The TR/Trx system has been implicated with a number of cellular processes including regulation of cell growth, apoptosis and the modification of the activity of transcription factors and receptors [4]. Mutant redox-inactive forms of Trx are incapable of stimulating cell growth or inhibiting apoptosis suggesting that Trx must be reduced to exert its effects on cell growth [5]. TR catalyses the NADPH-linked reduction of Trx and treatment of cells with the TR inhibitors doxorubicin or diaziquinone leads to an inhibition of ribonucleotide reductase activity, and inhibition of cell growth [6].

In addition to its growth promoting properties, TR also acts as an antioxidant either directly or through the action of thioredoxin. TR can reduce and detoxify lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides directly using NADPH as a cofactor [7]. In addition, TR is able to regenerate bioactivity in proteins inactivated by oxidative stress [8,9] and can also regenerate ascorbic acid from dehydroascorbate [10].

The expression of TR appears to be regulated through a number of factors including Se supply [11], redox state of the cell [3], oxidative stress [12] and also through activation of protein kinase C (PKC) [13]. Increases in Se supply and oxidative stress lead to increased expression of TR

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[10,12], whilst activation of PKC decreases the expression of the enzyme [13]. In the newborn primate lung oxygen appears to be an important factor in promoting an increased expression of the TR/Trx system [14].

The selenoenzyme glutathione peroxidase (GPX) is also considered to exert powerful antioxidant function in the cell cytoplasm [15], and the expression of GPX can be increased in situations of oxidative stress [16] and when selenium supply is increased [17].

The association between TR expression, cell growth and oxidative stress has led us to speculate that changes in TR expression may be important in human foetal development. If this were the case, it is also of relevance that in the UK and other countries the intake of selenium may be insufficient to sustain optimal expression of TR and other selenoenzymes [18].

In the present study we have examined TR concentration and TR activity, and GPX-1 activity, in human liver cytosol obtained from seven foetuses (gestational age 16–20 weeks) and five neonates (aged 1 day–15 weeks).

2. Materials and methods

2.1. Chemicals

All reagents were from Sigma (Dorset, UK).

2.2. Liver samples

Human liver tissue was obtained at autopsy from seven foetuses (16–20 week gestation), and six term neonates who survived up to between 1 day and 15 weeks postnatally. Postmortem time varied between 1 and 48 h after death. The study was approved by the Paediatric-Reproductive Medicine Ethics of Medical Research Sub-Committee of Lothian Health Board and the Ethics Committee of Tayside Health Board. Informed written consent was obtained from relatives prior to removing tissue.

2.3. Preparation of hepatic cytosols

Homogenisation of the tissue took place on ice, in 3 vols. of HEPES buffer (10 mM) (pH 7.4) containing 2-mercaptoethanol (3 mM) and sucrose (0.25 M), using a glass Potter-Elvehjem homogeniser with a motor-driven Teflon pestle. The homogenate was centrifuged at $10\,000\times g$ for 15 min, and the supernatant centrifuged at $100\,000\times g$ for 1 h. Aliquots of cytosol were snap-frozen on dry ice and stored at −80°C.

Prior to assay for TR activity, cytosols were treated using Centricon-10 concentrator tubes (Amicon, MA, USA) to remove mercaptoethanol (which interferes with the TR activity assay). Cytosol (200 μl) was dispensed into the concentrator tube, together with 180 μl of assay buffer (100 mM potassium phosphate, 50 mM potassium chloride, 10 mM EDTA, 0.2 mg/ml BSA; pH 7.0). The concentrator tube was then centrifuged at $5000\times g$ for 1 h. After centrifugation, the filtrate was removed and a further 180 μl of buffer was added to the sample, and the centrifugation step repeated. At the end of the procedure, all samples were made up to a volume of 200 μl using assay buffer.

2.4. Measurement of TR activity

TR activity was measured by the method of Hill et al. using DTNB (5,5′-dithiobis(2-nitrobenzoate)) as substrate in the presence and absence of 20 μM gold thioglucose [19], adapted for use on the Cobas FARA centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). All samples were measured in duplicate. Results were corrected for cytosol protein content, measured by the Bradford assay method [20] with BSA as standard.

2.5. Measurement of cytoplasmic GPX activity

GPX-1 activity was measured by the method of Paglia and Valentine [21], adapted for use on a Cobas FARA centrifugal analyser. All samples were measured in duplicate. Results were corrected for protein measured by the Bradford assay system.

2.6. Radioimmunoassay (RIA) of TR1

An in-house double-antibody RIA was used to measure TR1 concentration in hepatic cytosols. Human TR1 was purified from human placental cytosol as described by Holmgren and Björnstedt [22] and this was used to prepare standards, tracer and act as an immunogen to raise antiserum in rabbits. The tracer was ^{125}I-labelled human placental TR1 prepared using Bolton-Hunter reagent according to the manufacturer's instructions (Amersham, Bucks, UK).

For the assay 100 μl of tracer (approx. 15 000 cpm) was pipetted with 100 μl standard or sample. Anti-TR1 antibody (100 μl; initial dilution 1/30 000) was then added to all tubes which were incubated at 4°C overnight. The following day, pre-precipitated second antibody (donkey anti-rabbit serum) prepared as described previously [23] was added to each tube and incubated at room temperature with shaking for 1 h. After this second incubation step, 1.5 ml wash solution (0.05% v/v Brij 35 and 0.001% w/v microcrystalline cellulose) was added and the tubes centrifuged at $3000\times g$ for 30 min. The supernatants were then removed by decanting and the radioactivity in the pellet counted on a 1261 Multigamma Gamma Counter (Wallac, Gaithersburg, MD, USA). The standard curve was plotted and results interpolated using a multicalc data processing package (Wallac).

Results were corrected for protein measured by the Bradford method [20]. All samples and standards were

assayed in duplicate. The intra-assay precision of the TR radioimmunoassay was < 10% coefficient variation over the range of concentrations measured.

2.7. Statistical analysis

The significance of the differences in TR concentration, TR activity and GPX-1 activity between foetal and neonatal cytosols was tested using the *t*-test with Welch correction for unpaired data.

3. Results

3.1. Thioredoxin reductase activity and concentration in hepatic cytosols

The results are shown in Fig. 1 and Table 1. The median and interquartile (1st to 3rd) range of activity of TR was significantly greater ($P < 0.0005$) in foetal liver (2.05, 1.76–2.47 U/g protein) than in the neonatal liver (0.65, 0.44–0.74 U/g protein). Similarly the concentration of TR in foetal liver (43.56, 37.92–50.80 μg/g protein) was significantly higher ($P < 0.05$) than the concentration found in neonatal liver (11.59, 8.70–14.99 μg/g protein).

3.2. Cytoplasmic glutathione peroxidase activity in hepatic cytosols

The median and interquartile (1st to 3rd) range of GPX-1 activity in the foetal cytosols was 199.8 U/g protein (143.9–227.9), which was significantly greater ($P < 0.005$) than that found in the neonatal cytosols, 77.0 U/g protein (58.4–110.3) (Table 1).

There were strong correlations between GPX-1 activity and TR concentration ($r^2 = 0.58$; $P < 0.002$) and GPX-1 activity and TR activity ($r^2 = 0.4$; $P < 0.02$).

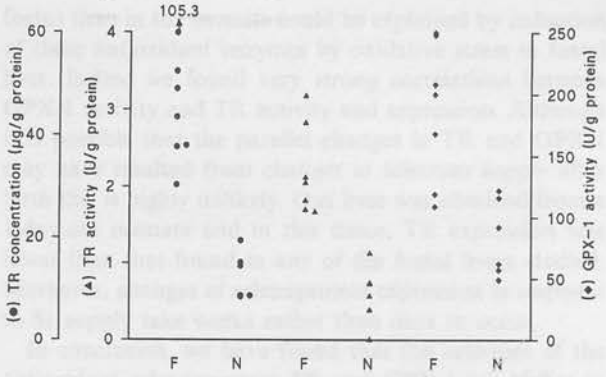

Fig. 1. Thioredoxin reductase concentration and activities, and cytoplasmic glutathione peroxidase activities of foetal and neonatal hepatic cytosols. TR concentration (●), TR activity (▲), and GPX-1 activity (♦) of foetal (F) and neonatal (N) cytosols are expressed as median values.

4. Discussion

We found that TR activity and concentration in human foetal liver are approx. 3-fold greater than in neonatal liver, with similar differences observed for hepatic cytoplasmic GPX activity.

These findings in the human contrast markedly with results reported in the rat where TR activity was found to increase progressively throughout the foetal, newborn and adult stages [24]. However, using immunohistochemistry one study in the rat reported that TR and Trx concentrations were higher in foetal and developing cells than in adult tissue [25]. The major enzymes of the glutathione redox cycle tend to increase in the liver of rats as the

Table 1
TR concentration and TR activities, and cytoplasmic GPX activities of foetal and neonatal hepatic cytosols

Gestational/postnatal age (weeks)	TR concentration (μg/g protein)	TR activity (U/g protein)	GPX-1 activity (U/g protein)
Foetal			
17/52	43.6	3.2	168.1
20/52	52.5	1.7	199.8
16/52	49.1	1.7	109.0
20/52	30.4	2.1	119.8
Unknown	38.1	2.1	247.7
16/52	105.3	1.8	250.0
16/52	37.8	2.8	208.1
Median (interquartile range)	43.6 (37.9–50.8)	2.1 (1.8–2.5)	199.7 (144.0–227.9)
Neonatal			
40/52+9	8.7	0.57	49.0
28/52+1 day	14.5	0.39	92.5
40/52+13	19.5	0.74	116.2
40/52+13	15.2	ND	61.5
40/52+15	8.7	1.13	121.9
40/52+5	8.5	0.73	57.3
Median (interquartile range)	11.6† (8.7–15.0)	0.65† (0.44–0.74)	77.0† (58.4–110.3)

ND, not detectable.
†$P > 0.05$ significant difference from foetal values (unpaired *t*-test with Welch correction).

animals develop through the foetal, neonatal and adult stages [26,27]. For example, GPX-1 activities in neonatal and adult rat liver are 2.4-fold and 13-fold greater respectively than in the foetus [26].

We can find no previous reports of TR ontogeny in humans but our results suggest that the rat model does not reflect the pattern observed in the human. Similar discrepancies have been found between rat and human for the ontogeny of other selenoenzymes in the liver, including type I and type III iodothyronine deiodinases [28]. Furthermore our observations suggest that the changes in selenoenzyme expression seen around birth in the rat may not be due to maturation of selenoenzyme expression but rather a physiological regulation process that is not yet fully understood.

We found that GPX-1 activity was approx. 3-fold higher in foetal than in neonatal liver. Asikaninen et al. [29] have reported that GPX-1 expression does not change significantly between the foetal and neonatal period in humans. The reasons for the discrepancy between our results and those of Asikaninen et al. are unclear, although their data for GPX-1 activities showed a markedly skewed distribution.

TR has many functions acting alone or in concert with thioredoxin. The TR/Trx system may modify cell growth [5], exhibit oncoprotein-like properties [30] and promote cell proliferation by increasing cellular resistance to apoptosis [5,31]. The association between TR expression and cell growth might thus suggest that changes in TR expression may provide a mechanism by which foetal and neonatal development is controlled.

Alternatively, changes in TR expression in the foetus and neonate may be linked to oxidative stress, modified redox state of the cell or changes in calcium signalling or selenium supply [14,32]. In baboon lung, TR is expressed constitutively at low levels in the foetus, and increases rapidly with the onset of O_2 or air breathing at birth [15]. Similarly the induction of GPX expression is frequently observed in situations where there is an increased oxidant stress; for example, thyroidal GPX-1 increases in iodine deficiency [17]. It has been suggested that changes in TR activity may be linked to the redox state of the cell, with a consequent effect on redox-regulated cell signalling [3]. These workers proposed that intracellular generation of reactive oxygen species oxidises the selenol group of TR, with a consequent decrease in enzymic activity. The resulting oxidation of Trx would then modulate Trx-dependent cellular constituents, including transcription factors (e.g. nuclear factor κB) and antioxidant enzymes (e.g. thioredoxin peroxidase). We have observed that TR activity and concentration change in parallel between foetal and neonatal liver, suggesting that this mechanism does not explain the differences in TR activity between the foetus and neonate.

Whilst TR may act as a growth factor, our observations that activities of both TR and GPX-1 are higher in the foetus than in the neonate could be explained by induction of these antioxidant enzymes by oxidative stress in foetal liver. Indeed we found very strong correlations between GPX-1 activity and TR activity and expression. Although it is possible that the parallel changes in TR and GPX-1 may have resulted from changes in selenium supply after birth this is highly unlikely. One liver was obtained from a 1-day-old neonate and in this tissue, TR expression was lower than that found in any of the foetal livers studied. Moreover, changes of selenoprotein expression in response to Se supply take weeks rather than days to occur.

In conclusion, we have found that the activities of the antioxidant selenoenzymes TR and GPX-1 are higher in the foetal than in the neonatal liver. We speculate that these differences may reflect altered states of oxidative stress during development.

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Selenoprotein expression in endothelial cells from different human vasculature and species

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Abstract

Selenium (Se) can protect endothelial cells (EC) from oxidative damage by altering the expression of selenoproteins with antioxidant function such as cytoplasmic glutathione peroxidase (cyGPX), phospholipid hydroperoxide glutathione peroxidase (PHGPX) and thioredoxin reductase (TR). If the role of Se on EC function is to be studied, it is essential that a model system be chosen which reflects selenoprotein expression in human EC derived from vessels prone to developing atheroma. We have used [^{75}Se]-selenite labelling and selenoenzyme measurements to compare the selenoproteins expressed by cultures of EC isolated from different human vasculature with EC isolated from bovine and porcine aorta. Only small differences were observed in selenoprotein expression and activity in EC originating from human coronary artery, human umbilical vein (HUVEC), human umbilical artery and the human EC line EAhy926. The selenoprotein profile in HUVEC was consistent over eight passages and HUVEC isolated from four cords also showed little variability. In contrast, EC isolated from pig and bovine aorta showed marked differences in selenoprotein expression when compared to human cells. This study firmly establishes the suitability and consistency of using HUVEC (and possibly the human cell line EAhy926) as a model to study the effects of Se on EC function in relation to atheroma development in the coronary artery. Bovine or porcine EC appear to be an inappropriate model.

Keywords: Selenium; Atherosclerosis; Endothelium; Selenoprotein

1. Introduction

The intake of selenium (Se) can to be inversely correlated with the incidence of atherosclerosis and coronary heart disease [1,2]. Oxidative damage to the endothelium is thought to be a primary event in the pathogenesis of atherosclerosis and Se, added as selenite, can protect the EC from such damage by altering the expression of specific selenoproteins [3,4]. The intracellular selenoproteins identified to date as having a potential antioxidant function include cytoplasmic glutathione peroxidase (cyGPX), phospholipid hydroperoxide glutathione peroxidase (PHGPX) and isoenzymes of thioredoxin reductase (TR).

Labelling cells with [^{75}Se]-selenite provides a sensitive method for assessing the expression of selenoproteins. Since equilibration of exogenous [^{75}Se]-selenite with the endogenous pool of Se and selenoproteins can take in excess of 24 h [5,6], such labelling experiments require incubation with [^{75}Se]-selenite for 36–48 h. Using these techniques to detect selenoproteins in human umbilical vein endothelial cells (HUVEC), TR has been identified as the predominant selenoprotein comprising 43% of the total selenoproteins [6]. Both cyGPX and PHGPX are also expressed by cultured EC obtained from a number of species including humans [4,7,8] but the relative activities of TR in EC isolated from different species have not been defined.

The culture of EC derived from the large vasculature is a well-established model for the study of the endothelium. Ideally, EC isolated from human coronary arteries would be preferred for studies relating to cardiovascular disease in man since atheroma is common in these vessels and is a major cause of morbidity and mortality. In practice, the human umbilical vein is often the chosen source of EC for the study of human endothelial function because of its accessibility.

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Moreover, it is a non-branching vessel with a large intimal surface area, making it technically easy to isolate cells. Unfortunately, the viability of isolated EC can be modified by several factors including foetal stress, maternal anaesthesia, smoking and other toxins [9,10]. The use of HUVEC and other primary cultures of EC is also complicated by genetic variability between preparations, limited population doublings and the requirement for specialised growth factors. Arterial and venous EC show differences in the production of angiotensin-converting enzyme [11] and their response to cytokine stimulation [12]. These observations have led to the suggestion that HUVEC, despite being widely used by researchers in the field of vascular disease, may not be the most suitable model for studying human cardiovascular disease [13–15].

Bovine aortic endothelial cells (BAEC) and porcine aortic endothelial cells (PAEC) are also often used as models to study EC function. PAEC may be a suitable alternative to HUVEC since there are similarities between the porcine and human cardiovascular system [16]. In addition, porcine aorta is subject to atheroma formation and has been used as a model for the study of this process [17]. However, variation between EC isolated from different species has been acknowledged to occur; for example, PAEC, unlike HUVEC and BAEC, do not express Factor VIII-related antigen [17].

The EC line EAhy926, established by hybridising primary HUVEC with A549 human lung tumour cells [18], has been used in a number of studies of EC function. EAhy926 retain many of the differentiated functions common to primary EC beyond 100 passages. These functions include: the expression of von Willebrand Factor [18]; prostacyclin formation [19]; expression of endothelin-1 [20]. The selenoprotein profile of EAhy926 cells has not been previously determined, but such work is essential in order to establish whether this convenient cell line would provide a suitable model for future studies of selenoprotein expression in EC.

If the role of Se on EC function is to be studied, it is essential that a model system be chosen which reflects the selenoprotein expression and function of human EC derived from vessels prone to developing atheroma. The experiments reported here use [^{75}Se]-selenite labelling and enzyme measurements to compare the selenoproteins expressed by cultures of EC isolated from different human vasculature with EC isolated form bovine and porcine aorta. The selenoprotein profile of the human EC cell line EAhy926 has also been studied.

2. Methods

2.1. Isolation and culture of EC

2.1.1. HUVEC and human umbilical artery endothelial cells (HUAEC)

Human umbilical cords (>100 mm in length) were obtained at normal deliveries or Caesarean section from nonsmoking women. Immediately after delivery, the cords were placed into sterile Earle's balanced salt solution (EBSS) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) and kept at 4 °C. EC were isolated within 20 h of delivery using a method adapted from that described previously by Anema et al. [6] and Jaffe et al. [21]. Cells were cultured in Endothelial Growth Medium-2 (EGM-2: Biowhittaker, Berkshire, UK) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) at 37 °C in an atmosphere of 5% CO_2, 95% air.

The cells showed the morphology characteristic of EC in culture previously described by Jaffe et al. [21]. Cells also synthesised von Willebrand Factor as determined by an indirect immuno-fluorescent detection system.

2.1.2. Human coronary arterial endothelial cells (HCAEC)

These were purchased from Biowhittaker, and tested positive for the presence of von Willebrand Factor and acetylated LDL (an alternative method for the specific characterisation of EC in culture) [22]. The HCAEC also displayed the characteristic morphology of EC. HCAEC were maintained in EGM-2 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml), and cultured at 37 °C in an atmosphere of 5% CO_2, 95% air.

2.1.3. EAhy926 EC line

EAhy926 cells were kindly donated by Professor Cora-Jean Edgell of the University of North Carolina, North Carolina, USA. The cells were maintained in high glucose (4.5 g/l) Dulbeco's Modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), 5 mM hypoxanthine , 0.02 mM aminopterin, 0.8 mM thymidine, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). The cells were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air and were passaged weekly using 0.25% trypsin–0.02% EDTA solution. EAhy926 cells displayed the characteristic morphology of EC in culture and stained positive for vWF.

2.1.4. BAEC

These were purchased from Biowhittaker. The certificate of analysis supplied with this product stated that the BAEC tested positive for acetylated LDL. The cells were maintained in EGM (Biowhittaker) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) and incubated at 37 °C in an atmosphere of 5% CO_2, 95% air.

2.1.5. PAEC

Porcine aorta was obtained within 5–10 min of slaughter from pigs aged under 2 years and was immediately placed into sterile EBSS at 4 °C containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). EC were isolated within 2–3 h of dissection using a method adapted from that previously described by Slater and Sloan [16]. Briefly, segments of about 5–10 cm were cut and any

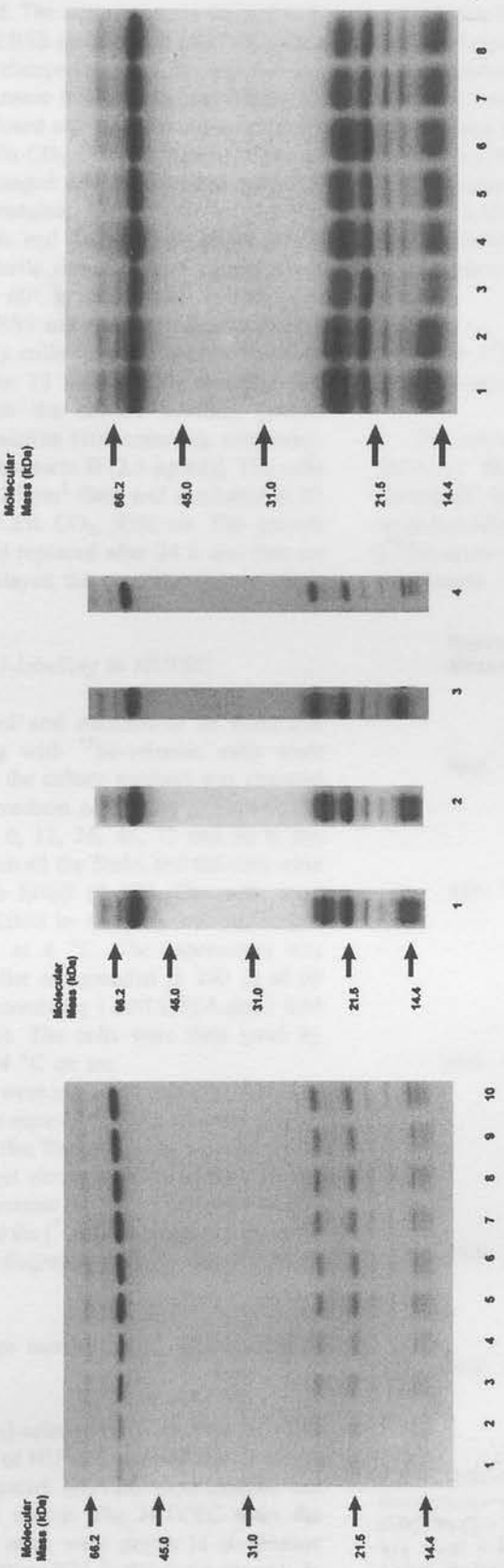

Fig. 1. Left. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of HUVEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for various lengths of time over a 96-h period. Duplicate flasks of HUVEC were labelled for each time point. Lanes 1, 2: 12 h; lanes 3, 4: 24 h; lanes 5, 6: 48 h; lanes 7, 8: 72 h; lanes 9, 10: 96 h. Each lane was loaded with 25-μg protein. Middle. Autoradiographs of four SDS-PAGE gels showing the intracellular selenoproteins from four different preparations of HUVEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Each lane was loaded with 25 μg of protein. Right. Autoradiograph of an SDS-PAGE gel of a single isolation of HUVEC at different passages each labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Lanes 1 to 8 represent passages 1 to 8, respectively. Each lane was loaded with 25 μg of protein.

minor vessels were ligated. The segments were washed with approximately 25 ml of EBSS (prewarmed to 37 °C). One end of the aorta was then clamped shut and the opposite end infused with 0.1% collagenase in EBSS (approximately 10 ml). This end was then closed and the cord incubated at 37 °C in an atmosphere of 5% CO_2, 95% air. After 15 min the segment was gently massaged and the contents collected into a sterile universal container. The vessel was then cut along its longitudinal axis and the luminal surface gently scraped gently with a sterile stainless steel scalpel blade angled at approximately 60° to the intimal surface. The blade was washed with EBSS and the washings added to the cell suspension previously collected. The sample was then centrifuged at $450 \times g$ for 10 min and the resulting cell pellet was washed with the culture medium [M199 containing 20% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml)]. The cells were resuspended into a 25-cm^2 flask and incubated at 37 °C in an atmosphere of 5% CO_2, 95% air. The growth medium was changed and replaced after 24 h and then on alternate days. Cells displayed the characteristic morphology of EC.

2.2. Time-course of [^{75}Se]-labelling in HUVEC

HUVEC were isolated and maintained as described previously. For labelling with ^{75}Se-selenite, cells were grown to confluence and the culture medium was changed and replaced with fresh medium containing [^{75}Se]-selenite (0.02 MBq/ml). At time 0, 12, 24, 48, 72 and 96 h, the medium was removed from all the flasks and the cells were washed three times with EBSS (4 °C). The cells were harvested into 20 ml of EBSS by scraping and centrifuged at $2000 \times g$ for 10 min at 4 °C. The supernatant was aspirated and the cell pellet resuspended in 200 μl of 60 mM Tris buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol (Tris buffer). The cells were then lysed by sonication whilst kept at 4 °C on ice.

Protein concentrations were measured using the Bradford assay [23] and the samples were diluted to a common protein concentration with Tris buffer. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis to separate the [^{75}Se]-labelled proteins present in 25 μg of protein. The resulting gel was dried and the [^{75}Se]-labelled selenoproteins were visualised by autoradiography using Kodak X-OMAT XAR-5 film.

2.3. The effect of passage number on [^{75}Se]-labelling in HUVEC

The intracellular [^{75}Se]-selenoprotein profiles of eight passages of a preparation of HUVEC isolated from a single umbilical vein were compared. HUVEC were isolated and maintained as described above. The HUVEC from the primary isolate (passage zero) were grown to confluence and passaged into at least three T75 flasks (passage one). At confluence, HUVEC in two of the T75 flasks were labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h, whilst the third was subcultured further to provide passage two. This procedure was continued until cells had reached passage eight, at which point distinct morphological changes were observed, such as significant cell enlargement and a partial loss of the characteristic cobblestone appearance.

After a 48-h labelling period, cells from each passage were harvested and stored at − 20 °C. Samples were thawed, lysed and prepared for separation using SDS-PAGE as described above.

2.4. The [^{75}Se]-labelling of vascular EC isolated from different vasculature

The intracellular selenoprotein profile of HUVEC, HCAEC, HUAEC, EAhy926, BAEC and PAEC were compared. Each cell type was isolated and maintained as previously described above and labelled for 48 h with [^{75}Se]-selenite (0.02 MBq/ml) prior to SDS-PAGE electrophoresis. For HUVEC, the selenoprotein profiles in

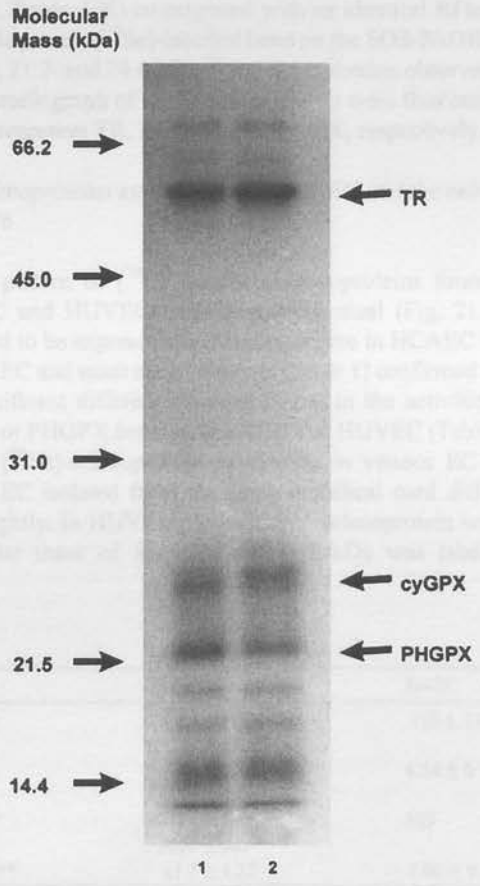

Fig. 2. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of HUVEC and HCAEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Lane 1, HUVEC; lane 2, HCAEC. Each lane was loaded with 25-μg protein. The selenoprotein bands representing cyGPX, PHGPX and TR are indicated.

four different preparations of cells isolated from four different cords were also compared.

2.5. Glutathione peroxidase and TR in EC

For each cell type the activities of TR, cGPX and PHGPX were measured in parallel subcultures of EC grown in triplicate 75-cm^2 flasks using the same culture medium as was used for the ^{75}Se labelling experiments. The activity of the selenoenzymes in EAhy926 cells was measured in cells grown in the DMEM culture medium specified above in the presence or absence of 40 nM selenite. After culture, cells were harvested by scrapping and lysed in 0.125 M potassium phosphate buffer (pH 7.4) by sonication on ice. TR concentration was measured by an in-house radioimmunoassay using antiserum raised against human placental TR. The activity of TR was determined using 5,5′ -dithiobis 2-nitrobenzoic acid (DTNB) as substrate in the presence and absence of 720 nM gold thioglucose [24]. The activities of cyGPX and PHGPX activity were determined as described previously [25].

2.6. Se content of culture medium

The Se content of the culture medium used for each cell type was determined using a fluorometric assay as described by Olsen et al. [26].

2.7. Statistical analysis

An unpaired Student's *t* test (with Welch correction when appropriate) was used to test for significant differences between the levels of TR, cyGPX and PHGPX.

3. Results

3.1. [^{75}Se]-labelling in HUVEC

Fig. 1 (left panel) shows the autoradiograph of a SDS-PAGE gel demonstrating the changes in [^{75}Se]-labelling over time. Selenoproteins were only faintly labelled after a 12-h exposure to [^{75}Se]-selenite. The intensity of labelling of all selenoproteins increased up until 48 h, at which time a steady state of labelling was achieved. Three major [^{75}Se]-labelled selenoproteins were observed with mean molecular masses of 58.1, 21.7 and 24.4 kDa. A selenoprotein of approximately 15 kDa was also moderately expressed. A number of minor labelled selenoproteins were observed.

No distinct variations in the pattern of intracellular [^{75}Se]-labelled selenoproteins between four different preparations of HUVEC were observed (Fig. 1, centre panel) and similarly the selenoprotein profile did not change over eight passages in HUVEC obtained form a single cord (Fig. 1, right panel).

Using antiserum to rat TR and human TR, we have previously identified by Western blotting the 58.1-kDa [^{75}Se]-labelled band in HUVEC as TR. Western blotting with antiserum to rat testis PHGPX demonstrated an immunoreactive band that co-migrated with the 21.7-kDa [^{75}Se]-labelled band. The same approach using antiserum to human cyGPX failed to visualise an immunoreactive band in HUVEC. However, purified human cyGPX (Sigma Chemical Co., Poole, UK) co-migrated with an identical Rf to the 24.4-kDa protein [^{75}Se]-labelled band on the SDS-PAGE gel. The 58-, 21.7- and 24.4-kDa ^{75}Se-selenoproteins observed on the autoradiograph of the SDS-PAGE gels were thus considered to represent TR, PHGPX and cyGPX, respectively.

3.2. Selenoproteins expressed by human EC and the cell line EAhy926

The pattern of [^{75}Se]-labelled selenoproteins found in HCAEC and HUVEC were almost identical (Fig. 2). TR appeared to be expressed to a higher degree in HCAEC than in HUVEC and mass measurements (Table 1) confirmed this. No significant difference was observed in the activities of cyGPX or PHGPX between HCAEC and HUVEC (Table 1).

The [^{75}Se]-selenoprotein expression in venous EC and arterial EC isolated from the same umbilical cord differed only slightly. In HUVEC an additional selenoprotein with a molecular mass of approximately 27 kDa was labelled,

Table 1
Selenoproteins expressed by EC and the EC line EAhy926

	HUVEC	HCAEC	EAhy926	BAEC
CyGPX activity (mU/mg protein)	86 ± 0.7	88 ± 0.03	23 ± 0.5** *158 ± 24.5**	115 ± 7.0
PHGPX activity (mU/mg protein)	7.15 ± 2.09	8.36 ± 0.01	4.41 ± 1.86 *12.5 ± 3.0*	4.54 ± 0.76
TR concentration (ng/mg protein)	721 ± 63	1245 ± 335*	871 ± 149 *1002 ± 267*	ND
TR activity (mU/mg protein)	8.9 ± 0.60	24.4 ± 0.34**	11.7 ± 1.37	1.06 ± 0.16**

Results for EAhy926 are given for cells grown in basal culture medium and results in italics are from cells grown in basal medium supplemented with 40 nM sodium selenite.
TR was not detectable (ND) in BAEC because of the lack of cross-reactivity of the antisera with bovine TR.
* Results significantly different from HUVEC, $P<0.05$.
** Results significantly different from HUVEC, $P<0.001$.

which was not observed in HUAEC. TR expression in HUAEC also appeared to be slightly greater than in HUVEC (Fig. 3).

The pattern of selenoprotein expression in EAhy926 cells closely resembled that observed in HUVEC (Fig. 4). The levels of TR and PHGPX in HUVEC were not significantly different to the levels found in EAhy926 cells grown in the basal medium or when supplemented with 40 nM selenite (Table 1). The activity of cyGPX in EAhy926 grown in basal medium was significantly lower than that found in HUVEC, however, EAhy926 cells supplemented with 40 nM selenite had significantly higher activities of cyGPX than HUVEC (Table 1). Supplementation with 40 nM selenite significantly ($P<0.05$) increased the expression of TR, cyGPX and PHGPX in EAhy926 cells.

3.3. Selenoprotein expression in BAEC and PAEC

Compared to HUVEC, PAEC showed quite distinct differences in the pattern of [^{75}Se]-labelled selenoproteins (Fig. 5, left panel). TR was not the most prominently labelled selenoprotein in PAEC but rather a selenoprotein of 15 kDa.

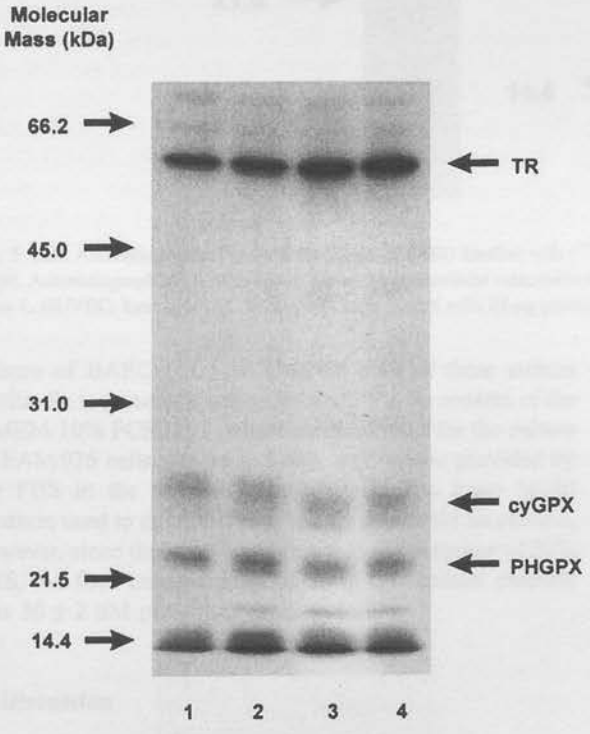

Fig. 3. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of HUVEC and HUAEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Both HUVEC and HUAEC were isolated from a single umbilical cord and samples were taken from duplicate flasks. Lanes 1 and 2, HUVEC; lanes 3 and 4, HUAEC. Each lane was loaded with 25-μg protein. The selenoprotein bands representing cyGPX, PHGPX and TR are indicated.

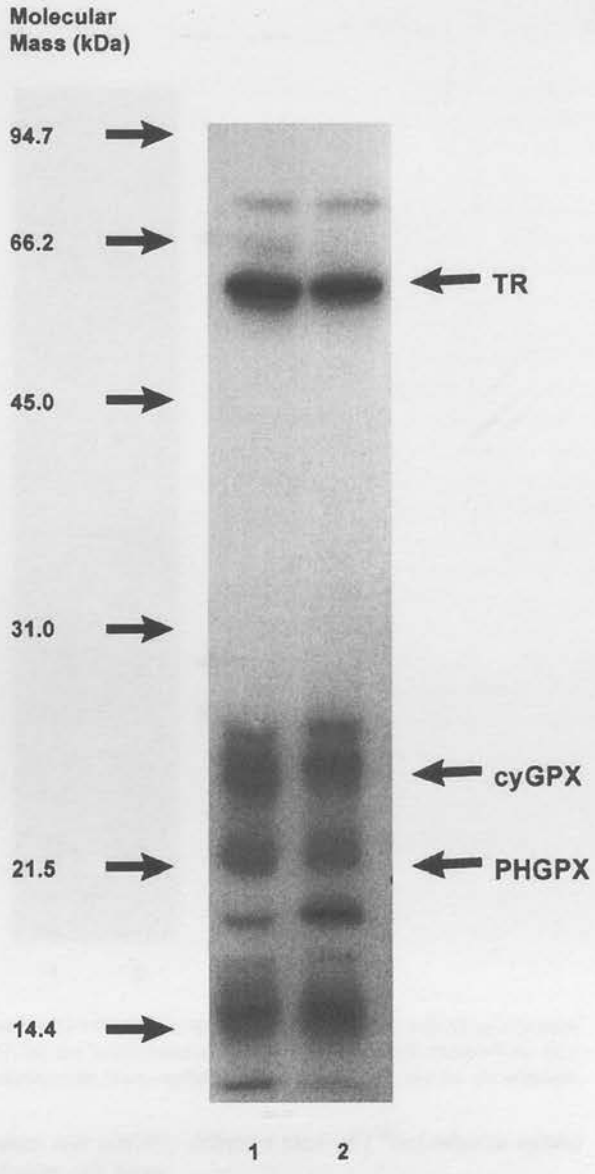

Fig. 4. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of HUVEC and EAhy926 cells labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Lane 1, HUVEC; lane 2, EAhy926 cells. Both lanes were loaded with 25-μg protein. The selenoprotein bands representing cyGPX, PHGPX and TR are indicated.

The [^{75}Se]-labelling of many selenoproteins in BAEC was significantly less than in HUVEC (Fig. 5, right panel). In particular, the expression of the TR band was much less in BAEC than HUVEC, an observation that was confirmed by activity measurements of TR (Table 1).

3.4. Se content of culture medium

The EGM2 used for the culture of HUVEC, HCAEC and HUAEC had a Se content of 30 ± 2 nM, which was not significantly different to the Se content of EGM used for the

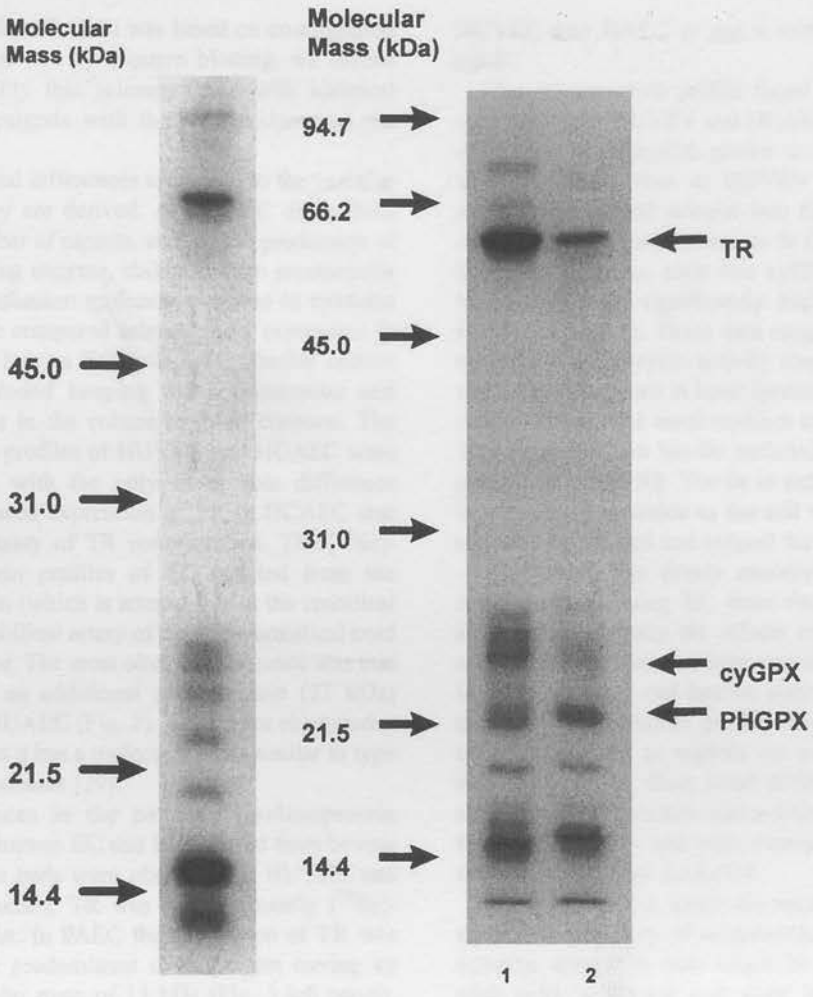

Fig. 5. Left. Autoradiograph of an SDS-PAGE gel of PAEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. The lane was loaded with 25 μg of protein. Right. Autoradiograph of an SDS-PAGE gel of the intracellular selenoproteins of HUVEC and BAEC labelled with [^{75}Se]-selenite (0.02 MBq/ml) for 48 h. Lane 1, HUVEC; lane 2, BAEC. Both lanes were loaded with 25-μg protein. The selenoprotein bands representing cyGPX, PHGPX and TR are indicated.

culture of BAEC (43 ± 10 nM). In both of these culture media, Se is present as selenious acid. The Se content of the DMEM/10% FCS/HAT culture medium used for the culture of EAhy926 cells was 18 ± 2 nM, with the Se provided by the FBS in the form of selenoprotein. The basal M199 medium used to culture PAEC had no detectable Se content, however, since these cells are grown in the presence of 20% FBS, the final concentration of Se in this culture medium was 36 ± 2 nM present as selenoprotein.

4. Discussion

These studies indicate that for HUVEC, an incubation period of at least 48 h must be employed to achieve maximal ^{75}Se labelling of selenoproteins (Fig. 1, left panel). This contrasts with human thyrocytes where [^{75}Se]-labelling reaches a steady state by 27 h [5]. This difference may be due to the variability in selenoprotein turnover between tissues and possibly different rates of [^{75}Se]-selenite uptake between cell types.

In primary culture, HUVEC have a limited replication potential, tending to senesce [27] and, in addition, the activities of some enzymes have been reported to vary with successive passages [28]. We observed no differences in the [^{75}Se]-selenoprotein labelling of HUVEC throughout eight passages of the same preparation under identical growth conditions (Fig. 1, right panel), arguing that the expression of selenoproteins in HUVEC remains constant over at least eight passages. In addition, we found that the selenoprotein profile in four individual preparations of HUVEC showed no marked variability between preparations (Fig. 1, centre). We have also confirmed our previous observation that HUVEC show dominant expression of the 58.1-kDa selenoprotein, TR [6]. Two other major [^{75}Se]-selenoproteins expressed by HUVEC, which were labelled to a much lesser extent than TR, had molecular masses of 21.7 and 24.4 kDa and were provisionally identified as cyGPX and PHGPX,

respectively. Since identification was based on co-migration with purified material and by Western blotting, we cannot exclude the possibility that selenoproteins with identical molecular mass co-migrate with these peroxidases on the SDS-PAGE system.

EC show functional differences according to the vascular bed from which they are derived. Arterial EC differ from venous EC in a number of aspects, such as the production of angiotensin-converting enzyme, ability to form prostacyclin [11] and their cell adhesion molecule response to cytokine stimulation [12]. We compared selenoprotein expression in a range of primary human EC cells using similar culture conditions that included keeping the concentration and chemical form of Se in the culture medium constant. The [^{75}Se]-selenoprotein profiles of HUVEC and HCAEC were remarkably similar, with the only observable difference being a slight increased expression of TR in HCAEC that was confirmed by assay of TR concentration. The [^{75}Se]-labelled selenoprotein profiles of EC isolated from the human umbilical vein (which is arterial-like in the umbilical cord) and human umbilical artery of the same umbilical cord were also very similar. The most obvious difference was that HUVEC expressed an additional selenoprotein (27 kDa) when compared to HUAEC (Fig. 2). We did not characterise this selenoprotein but it has a molecular mass similar to type I iodothyronine deiodinase [29].

Distinct differences in the pattern of selenoprotein expression between human EC and EC isolated from bovine and porcine vascular beds were observed. In HUVEC and other human EC studied, TR was the dominantly [^{75}Se]-labelled selenoprotein. In PAEC the expression of TR was relatively weak, the predominant selenoprotein having an approximate molecular mass of 15 kDa (Fig. 5 left panel). This 15-kDa [^{75}Se]-selenoprotein was also found in HUVEC but labelled to a lesser extent. The identity of the 15-kDa selenoprotein was not established, although its electrophoretic mobility is consistent with it being either the uncharacterised 15-kDa selenoprotein described by Gladyshev et al. [30] or the Se-containing protein, epidermal fatty acid-binding protein (E-FABP) [30]. Masouyé et al. [31] have demonstrated the presence of E-FABP in HUVEC but they were unable to detect the protein in the endothelium of the umbilical vein from which the EC were isolated, suggesting that the expression of E-FABP may be induced by cell culture.

The pattern of [^{75}Se]-selenoprotein expression in BAEC also differed considerably from that observed in HUVEC (Fig. 5, right panel). The [^{75}Se]-labelling of the majority of selenoproteins was significantly lower in BAEC compared to HUVEC and TR expression and activity was considerably lower in BAEC than in HUVEC (Table 1). The expression of selenoproteins is modified by the Se status of the cell. However, the Se content and the chemical form of Se (ie selenious acid) present in the culture medium for HUVEC (EGM2) and BAEC (EGM) were identical. This suggests that differing selenoprotein profiles seen in HUVEC and BAEC is not a consequence of altered Se status.

The selenoprotein profile found for EAhy926 cells was very similar to HUVEV and HCAEC, however, the activity of cyGPX in EAhy926 grown in basal medium was significantly lower than in HUVEV or HCAEC (Table 1). Inclusion of 40 nM selenite into the basal culture medium resulted in significant increases in the expression of each of the selenoenzymes, such that cyGPX activity increased to levels that were significantly higher than that found in HUVEC (Table 1). These data suggest that the small differences in selenoenzyme activity observed between HUVEC and EAhy926 grown in basal medium are due to the limiting supply of Se in the basal medium used to culture EAhy926. This basal medium has Se included as selenoprotein incorporated into the FBS. The Se in extracellular selenoproteins is not readily available to the cell whilst selenite is rapidly taken up by the cell and utilised for selenoprotein synthesis.

This study has firmly established the suitability and consistency of using EC from the human umbilical vein as a model to study the effects of Se on EC function in relation to atheroma development in the coronary artery. EC isolated from pig and bovine aorta showed marked differences in selenoprotein profile when compared to human cells, particularly as regards the expression of the antioxidant enzyme TR. Only small differences were observed in selenoprotein expression and activity in EC originating from the coronary artery and other various human vasculature and the human cell line EAhy926.

We conclude that whilst the most appropriate cell culture model for the study of selenoprotein expression in atherosclerotic disease in man might be HCAEC, the supply of such cells is limited and even then the cells are often isolated from diseased vessels. Our studies suggest that HUVEC and possibly EAhy926 cells are suitable alternative model systems to HCAEC in which the role of selenoproteins in protecting against atheroma formation can be studied.

Acknowledgements

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Selenite protects human endothelial cells from oxidative damage and induces thioredoxin reductase

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ABSTRACT

The ability of selenium to protect cultured human coronary artery endothelial cells (HCAEC), human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) from oxidative damage induced by 100 μM t-butyl hydroperoxide (t-BuOOH) was compared. Preincubation of human endothelial cells for 24 h with sodium selenite at concentrations as low as 5 nM provided significant protection against the harmful effects of 100 μM t-BuOOH, with complete protection being achieved with 40 nM selenite. The preincubation period was required for selenite to exert this protective effect on endothelial cells. When compared with selenium-deficient cells, the activities of cytoplasmic glutathione peroxidase (GPX-1), phospholipid hydroperoxide glutathione peroxidase (GPX-4) and thioredoxin reductase (TR) were each induced approx. 3–4-fold by 40 nM selenite. HCAEC and HUVEC showed great similarity in their relative abilities to resist oxidative damage in the presence and absence of selenite, and the activities of TR and the GPXs were also similar in these cell types. BAEC were more susceptible to damage by 100 μM t-BuOOH than were human endothelial cells, and could not be protected completely by incubation with selenite at concentrations up to 160 nM. The activity of TR in human endothelial cells was approx. 25-fold greater than that in BAEC of a similar selenium status, but GPX-1 and GPX-4 activities were not significantly different between the human and bovine cells. These studies, although performed with a small number of cultures, show for the first time that selenium at low doses can provide significant protection of the human coronary artery endothelium against damage by oxidative stress. TR may be an important antioxidant selenoprotein in this regard, in addition to the GPXs. The data also suggest that HUVEC, but not BAEC, represent a suitable model system in which to study the effects of selenium on the endothelium of human coronary arteries.

INTRODUCTION

Endothelial cells (EC) are constantly exposed to the possibility of oxidative damage from reactive oxygen species, such as superoxide, hydroxyl radicals, hydrogen peroxide, lipid peroxides and singlet oxygen. Oxidative damage to the endothelium is considered to be one of the principle mechanisms in the pathogenesis of athero-

Key words: endothelium, glutathione peroxidase, oxidative damage, selenium, thioredoxin reductase.

Abbreviations: BAEC, bovine aortic endothelial cells; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EBSS, Earl's balanced salt solution; EC, endothelial cells; EGM, endothelial growth medium; GPX, glutathione peroxidase; HCAEC, human coronary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; t-BuOOH, t-butyl hydroperoxide; TR, thioredoxin reductase.

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sclerosis [1–3]. Cytoprotection against oxidative damage is generally the function of vitamins and enzymes with antioxidant actions. These antioxidant enzymes include superoxide dismutases, catalase and a range of selenoenzymes, such as the glutathione peroxidases (GPXs) [4].

There is some evidence to suggest that selenium (Se) may prevent oxidative damage to EC, and thus protect against the development of atherosclerosis. For example, in humans low plasma Se levels have been associated with an increased risk of cardiovascular disease, including coronary atherosclerosis [4–6]. In addition, cultures of bovine aortic EC (BAEC) pretreated with sodium selenite are more resistant to oxidative damage induced by t-butyl hydroperoxide (t-BuOOH) or photogenerated oxidized low-density lipoprotein than are cells grown in Se-deficient medium [7]. This acquired resistance to oxidative damage in BAEC treated with selenite was attributed to increased activities of the selenoenzymes cytoplasmic GPX (GPX-1) and phospholipid hydroperoxide GPX (GPX-4) [7]. However, the involvement of other selenoenzymes with antioxidant actions, such as thioredoxin reductase (TR), was not investigated, since, at the time of the study, TR was not known to be a selenoenzyme [8].

Ideally, EC isolated from human coronary arteries (HCAEC) would be the preferred model for studies relating to coronary atheromatous disease in humans. In practice, aortic EC isolated from animals are widely used in model systems; alternatively, EC isolated from the human umbilical vein (HUVEC) are studied because of the accessibility of such tissues. However, HCAEC and HUVEC show differences in their production of angiotensin-converting enzyme [9] and their response to cytokine stimulation [10]. These observations have led to the suggestion that HUVEC may not be the most suitable model for studying human cardiovascular disease [11–13]. Similarly, we have found that the pattern of selenoprotein expression in ^{75}Se-labelled BAEC is clearly different from the pattern expressed in HUVEC (S. Miller, S. W. Walker and G. J. Beckett, unpublished work), an observation which suggests that the published data regarding the ability of Se to confer resistance to oxidative stress in BAEC may not be applicable to the human situation.

EC isolated from different vascular beds and from different species may thus show clear differences in their ability to resist oxidative stress in response to Se supplementation. Although previous work using BAEC [7] may be of relevance to humans, it is essential to determine if Se can exert a similar antioxidant response in HCAEC. Since it is now known that TR is a selenoenzyme with an antioxidant action [8], it is also important to establish if TR can be induced in HCAEC by concentrations of Se that are able to confer significant antioxidant effects.

In the present study we have thus used HCAEC, HUVEC and BAEC to determine if these cell types show differences in their ability to resist oxidative stress in the presence and absence of Se supplementation. We have also monitored the changes that occur in the activities of the three main antioxidant selenoenzymes, GPX-1, GPX-4 and TR, in response to small changes in Se supply. These studies were performed to investigate whether or not Se exerts important antioxidant actions on human EC, as is the case with BAEC. In addition, it was hoped to obtain evidence that TR is a potential mediator of the antioxidant actions of Se in human EC.

METHODS

Isolation and culture of HUVEC

Human umbilical cords (> 100 mm in length) were obtained at normal delivery or Caesarean section from non-smoking women. Immediately after delivery, the cords were placed into sterile Earl's balanced salt solution (EBSS) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml), and kept at 4 °C. EC were isolated within 20 h of delivery using a method adapted from that described previously [14,15]. Briefly, the umbilical vein was cannulated with a Venflon cannula (gauge 17/45 mm), which was then clamped into place. The vein was washed with 100 ml of EBSS (prewarmed to 37 °C) to remove any blood clots, and the outside was wiped using sterile gauze. One end of the cord was clamped shut and the opposite end was infused with 0.07 % (w/v) collagenase in EBSS (5–15 ml). The cord was then incubated at 37 °C in an atmosphere of 5 % CO_2/95 % air.

After 10 min the cord was removed and massaged gently. The contents of the cord were flushed out with 30 ml of Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution. The resulting cell suspension was collected and centrifuged at 450 *g* for 10 min, and the cell pellet was washed once with endothelial growth medium-2 (EGM-2: Biowhittaker Ltd, Wokingham, Berks., U.K.) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). The cells were resuspended in 15 ml of EGM-2 and plated out into one 75 cm^2 flask. This flask was then incubated at 37 °C in an atmosphere of 5 % CO_2/95 % air. After approx. 5 h the HUVEC were washed with 2 × 10 ml of EGM-2 to remove any blood, contaminant cells and cell debris. The medium was replaced with a further 15 ml of EGM-2, which was replenished on alternate days during the culture period.

When the cells were approx. 90 % confluent, they were passaged into 12-well plates (for cellular integrity studies) or 75 cm^2 flasks (for enzyme measurements) containing Se-deficient medium to which had been added sodium selenite at concentrations ranging from 0 to 160 nM. The

Se-deficient medium comprised M199 (Biowhittaker Ltd) supplemented with EGM-2 supplements [foetal bovine serum (2%), hydrocortisone (0.04%), ascorbic acid (0.1%), long R insulin-like growth factor-1 (0.1%), heparin (0.1%), human fibroblast growth factor (0.4%), human recombinant vascular endothelial growth factor (0.1%), human recombinant epidermal growth factor (0.1%) and gentamicin sulphate/amphotericin B (0.1%)]. The Se content of the M199 culture medium containing the EGM-2 supplements was 4.7 nM, as determined by acid digestion followed by fluorimetric analysis [16]. The low but detectable Se content in the complete medium is likely to be due to selenoproteins present in the foetal bovine serum, since no Se could be detected in the M199 culture medium in the absence of EGM-2 supplements.

The cells showed the morphology characteristic of EC in culture, as described previously by Jaffe et al. [15]. Under the light microscope, cells were non-overlapping, large and polygonal. After 3–7 days in culture, a confluent single monolayer of contact-inhibited cells with a cobblestone appearance was apparent. Cells also synthesized von Willebrand factor, as determined by an indirect immunofluorescent detection system, characteristic of EC in culture [15,17].

Culture of HCAEC

HCAEC were purchased from Biowhittaker UK Ltd, and tested positive for the presence of von Willebrand factor and the uptake of acetylated low-density lipoprotein (an alternative method for the specific characterization of EC in culture) [18]. The HCAEC also displayed the characteristic morphology of EC, as described above.

HCAEC were initially maintained in EGM-2 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml), and were cultured at 37 °C in an atmosphere of 5% CO_2/95% air. Subculture of cells with various concentrations of sodium selenite (0–160 nM) was carried out in M199 with EGM-2 supplement additions, as described above for HUVEC.

Culture of BAEC

BAEC were purchased from Biowhittaker UK Ltd. The certificate of analysis supplied with this product stated that the BAEC tested positive for acetylated low-density lipoprotein. The cells were maintained in EGM-2 containing penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml), and were incubated at 37 °C in an atmosphere of 5% CO_2/95% air. Subculture of cells with various concentrations of sodium selenite was carried out in M199 with EGM-2 supplement additions.

Determination of cellular integrity by measurement of lactate dehydrogenase (LDH) retention

Cell integrity was assessed as the percentage retention of LDH by the cell layer. Intracellular LDH activity and LDH in the culture medium was determined using an LDH kit method (Sigma Diagnostics Ltd, Poole, Dorset, U.K.) adapted for use on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, U.K.).

All EC for cell integrity studies were plated into 12-well plates at a density of 10000 cells/cm^2 and cultured for 5–7 days until confluent, using two changes of M199-based culture medium containing specified concentrations of selenite as described above. At confluence, the culture medium was replaced with fresh medium (1 ml) containing 100 μM t-BuOOH (and specified concentrations of selenite) and incubated for 20 h. The culture medium was then removed and kept, and the cells were washed with 2 × 1 ml of PBS, pH 7.4. The cells were lysed in 0.5 ml of 0.5% Triton X-100 (in PBS). After 15 min the cell lysates were collected and the wells were washed with a further 0.5 ml of PBS; the washings were combined with the respective lysates. Cell debris in the culture medium and cell lysates was removed by centrifugation of all samples at 11500 *g* for 10 min prior to assay for LDH activity.

LDH activity was also measured in culture media that had not been in contact with cells, as a measure of endogenous LDH in the culture medium (blank).

Protection of EC from t-BuOOH toxicity by Se

The protective effects of sodium selenite against oxidative damage caused by exposure to 100 μM t-BuOOH for 20 h was determined by measurement of LDH retention, as described above. Cells grown in M199 containing added concentrations of selenite ranging from 0 to 160 nM were used for these experiments.

To examine the importance of a preincubation period with selenite, LDH retention in response to 100 μM t-BuOOH for 20 h was also studied using cells grown in Se-deficient medium, but where selenite (at the specified concentration) and 100 μM t-BuOOH were added simultaneously. This protocol ensured that cells were not preincubated with selenite, as was the case for all other experiments.

All determinations were carried out in triplicate wells.

Effects of Se on selenoenzyme levels in EC

The effects of sodium selenite on the concentration and activity of TR and the activities of GPX-1 and GPX-4 were investigated in parallel cultures of EC (grown in

triplicate 75 cm^2 flasks) used for the toxicity studies described above. After culture, cells were harvested by scraping and lysed in 0.125 M potassium phosphate buffer (pH 7.4) by sonication on ice. The levels of the selenoenzymes were then determined as described below.

Measurement of selenoenzymes

TR concentration

The TR concentration was measured by an 'in-house' RIA using a primary antibody raised in rabbits to human placental TR. Second-antibody precipitation was employed, using pre-precipitated donkey anti-rabbit reagent, prepared by mixing 25 ml of donkey anti-rabbit serum with 1.5 ml of normal rabbit serum (Scottish Antibody Production Unit, Carluke, Scotland, U.K.) for 12 h at room temperature. After washing, the precipitate was made up to a final volume of 100 ml with assay diluent of 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 % BSA, 0.02 % sodium azide and 10 mM dithiothreitol.

The tracer was ^{125}I-labelled human placental TR, prepared using Bolton–Hunter reagent (Amersham International plc). Standards were prepared using purified placental human TR diluted in foetal bovine serum (Gibco, Paisley, Scotland, U.K.)

The RIA was performed using duplicates of samples and standards as follows. Standard or sample (100 μl) was added with 100 μl of ^{125}I-TR tracer (10000 d.p.m.; 50 pg per tube) and primary antibody (100 μl). After an overnight incubation at 4 °C, donkey anti-rabbit reagent (100 μl) was added. After a further 1 h at room temperature with shaking, wash solution (0.05 % Brij solution) was added to each tube, followed by centrifugation for 30 min at 1800 *g* (4 °C). The supernatant was decanted and the precipitate was washed with a further 1.5 ml of wash solution. The ^{125}I radioactivity in the precipitate was counted in a multi-well γ-radiation counter, and results were interpolated using the LKB 1224-RIACalc RIA evaluation program.

TR activity

TR activity was measured using a method adapted from that described previously by Hill et al. [19], based on the ability of TR to perform the NADPH-dependent reduction of the substrate 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to produce 5′-thionitrobenzoic acid. TR activity was determined by following the rate of DTNB reduction, measured as an increase in absorbance at 412 nm, using a Cobas Fara Centrifugal Analyser (Roche Diagnostics). To correct for the reduction of DTNB by substances other than TR, the assay was performed in the presence and absence of 720 nM aurothioglucose, a concentration that inhibits TR activity.

GPX-1 and GPX-4 activities

GPX-1 activity was determined by following the rate of NADPH oxidation at 340 nm in the presence of the substrate hydrogen peroxide, using a Unicam UV4 spectrometer with Vision software [20]. GPX-4 activity was measured using the same system as for GPX-1, but with phosphatidylcholine hydroperoxide as substrate [20].

Statistical analysis

One-way ANOVA was used to test for significant differences in LDH retention in response to t-BuOOH. A Tukey–Kramer multiple-comparison post-hoc test was used to test for significant differences in LDH retention in response to t-BuOOH toxicity between cells with differing Se status. These statistical tests were also used to test for significant differences between levels of selenoproteins in EC cultured in different concentrations of sodium selenite.

RESULTS

Protection by Se of EC from t-BuOOH toxicity

Preliminary experiments demonstrated that exposure to 100 μM t-BuOOH for 20 h consistently produced a significant ($P < 0.001$) decrease in LDH retention by EC, to values that were < 20 % of those in control cells not exposed to t-BuOOH. HCAEC (Table 1) and HUVEC (Table 2) had a similar sensitivity to 100 μM t-BuOOH, with approx. 13 % retention of LDH found in both of these cell types at the end of the 20 h incubation. In contrast, BAEC (Table 3) were significantly ($P < 0.01$) more sensitive to 100 μM t-BuOOH than human EC, retaining only approx. 3 % of LDH after exposure to the hydroperoxide.

In each of the three HUVEC and two HCAEC cultures, preincubation with 40 nM sodium selenite achieved complete protection against the damaging effects of t-BuOOH (Tables 1 and 2). In three of the five experiments, significant but not optimal protection by Se was achieved at a sodium selenite concentration of 10 nM; in one experiment using HCAEC, significant protection was observed with 5 nM sodium selenite (Table 2). In each of the two experiments using BAEC, selenite afforded significant protection against the toxic effects of 100 μM t-BuOOH (Table 3). However, although 40 nM selenite afforded optimal protection to BAEC, this was by no means complete; increasing the concentration of selenite to 160 nM provided no additional protection.

Preincubation with sodium selenite was required for protection against t-BuOOH toxicity. No protective

Table 1 Changes in selenoenzyme expression and resistance to oxidative damage caused by t-BuOOH in HCAEC supplemented with sodium selenite

HCAEC were cultured in Se-deficient medium or the same medium supplemented with specified concentrations of sodium selenite until confluent in 12-well plates (LDH retention studies) or 75 cm^2 flasks (enzyme studies). LDH retention was measured in response to exposure to 100 μM t-BuOOH for 20 h. TR, GPX-1 and GPX-4 were determined as specified in the Methods section. All incubations were carried out in triplicate. Significant differences from Se-deficient cells: $^*P < 0.05$; $^{***}P < 0.001$.

Sodium selenite (nM)	LDH retention (%)		TR mass (ng/mg)	TR activity (m-units/mg)	GPX-1 activity (units/mg)	GPX-4 activity (m-units/mg)
	0 μM t-BuOOH	100 μM t-BuOOH				
Prep. 1						
0	96.8 ± 13	12.8 ± 1.3	913 ± 30	6.4 ± 0.04	0.037 ± 0.011	8.36 ± 0.01
1	97.1 ± 1.0	7.9 ± 0.9	828 ± 35	6.3 ± 0.44	0.049 ± 0.005	5.98 ± 4.11
5	94.6 ± 0.5	8.7 ± 0.8	1111 ± 10***	11.7 ± 0.08***	0.057 ± 0.004	9.65 ± 2.97
10	97.6 ± 0.6	40.4 ± .9.2***	1620 ± 37***	17.1 ± 0.63***	0.090 ± 0.020*	9.78 ± 3.52
40	99.2 ± 1.0	94.6 ± 0.4***	1716 ± 15***	24.4 ± 0.34***	0.121 ± 0.013***	18.05 ± 7.26
160	99.2 ± 1.0	95.1 ± 0.8***	1059 ± 110***	25.9 ± 0.08***	0.148 ± 0.033***	16.54 ± 1.56
Prep. 2						
0	93.0 ± 0.6	12.6 ± 1.1	823 ± 41	7.2 ± 0.06	0.088 ± 0.031	5.89 ± 2.72
1	92.9 ± 0.3	9.9 ± 1.9	956 ± 35	9.9 ± 0.41	0.070 ± 0.001	3.61 ± 4.46
5	93.9 ± 0.4	8.9 ± 3.5	1437 ± 128***	20.6 ± 2.9***	0.120 ± 0.011	10.84 ± 4.64
10	93.4 ± 0.6	92.2 ± 0.5***	1659 ± 131***	24.4 ± 1.4***	0.138 ± 0.037	15.48 ± 2.58
40	93.2 ± 0.9	90.6 ± 0.4***	1756 ± 96***	26.3 ± 2.3***	0.118 ± 0.012	16.84 ± 4.09
160	94.1 ± 0.2	89.6 ± 2.1***				

Table 2 Changes in selenoenzyme expression and resistance to oxidative damage caused by t-BuOOH in HUVEC supplemented with sodium selenite

HUVEC were cultured in Se-deficient medium or the same medium supplemented with specified concentrations of sodium selenite until confluent in 12-well plates (LDH retention studies) or 75 cm^2 flasks (enzyme studies). LDH retention was measured in response to exposure to 100 μM t-BuOOH for 20 h. TR, GPX-1 and GPX-4 was determined as specified in the Methods section. All incubations were carried out in triplicate. Significant differences from Se-deficient cells: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Sodium selenite (nM)	LDH retention (%)		TR mass (ng/mg)	TR activity (m-units/mg)	GPX-1 activity (units/mg)	GPX-4 activity (m-units/mg)
	0 μM t-BOOH	100 μM t-BOOH				
Prep. 1						
0	91.3 ± 1.3	16.9 ± 1.6	523 ± 64	3.6 ± 0.9	0.032 ± 0.093	4.18 ± 2.09
10	91.4 ± 1.2	25.6 ± 8.0	662 ± 43***	7.4 ± 0.4***	0.111 ± 0.021***	15.26 ± 3.85**
40	91.5 ± 0.6	91.4 ± 1.3***	805 ± 71***	8.9 ± 0.6***	0.170 ± 0.027 ***	11.90 ± 3.81
160	93.2 ± 0.8	91.9 ± 0.8***	790 ± 36***	8.7 ± 0.2***	0.164 ± 0.010***	13.46 ± 2.28*
Prep. 2						
0	94.5 ± 0.5	11.3 ± 0.7	441 ± 34	2.4 ± 0.1		
1	94.5 ± 0.5	8.2 ± 0.6	472 ± 30	3.2 ± 0.2*		
5	93.1 ± 1.1	80.1 ± 2.0***	642 ± 47***	5.7 ± 0.2***		
10	92.6 ± 1.9	84.0 ± 4.8***	801 ± 22***	7.9 ± 0.4***		
40	93.8 ± 0.7	93.4 ± 0.3***	895 ± 92***	9.5 ± 0.3***		
Prep. 3						
0	93.1 ± 0.7	22.7 ± 2.2	345 ± 59	1.4 ± 0.1		
1	92.8 ± 0.4	19.8 ± 2.7	390 ± 120	1.8 ± 0.1		
5	91.3 ± 0.7	11.6 ± 0.8	489 ± 40	3.2 ± 0.5*		
10	91.2 ± 0.1	89.1 ± 0.9***	644 ± 38**	3.7 ± 0.8***		
40	89.8 ± 0.9	86.3 ± 2.3***	1277 ± 62***	7.2 ± 0.6***		
160	90.6 ± 0.5	89.2 ± 0.4***	1059 ± 110***	8.7 ± 1.2***		

Table 3 Changes in TR activity and resistance to oxidative damage caused by t-BuOOH in BAEC supplemented with sodium selenite

BAEC were cultured in Se-deficient medium or the same medium supplemented with specified concentrations of sodium selenite until confluent in 12-well plates (LDH retention studies) or 75 cm^2 flasks (enzyme studies). LDH retention was measured in response to exposure to 100 μM t-BuOOH for 20 h. TR was determined as specified in the Methods section. All incubations were carried out in triplicate. Significant differences from Se-deficient cells: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. ND, not detectable.

	LDH retention (%)		
Sodium selenite (nM)	0 μM t-BuOOH	100 μM t-BuOOH	TR activity (m-units/mg)
Prep. 1			
0	100.7 ± 1.3	4.4 ± 0.8	ND
1	100.3 ± 0.5	4.0 ± 1.2	0.17 ± 0.20
5	99.3 ± 0.5	8.4 ± 3.2	0.98 ± 0.22
10	99.2 ± 0.3	3.9 ± 0.2	1.03 ± 0.13***
40	100.6 ± 0.1	65.8 ± 7.5***	1.06 ± 0.16***
160	99.3 ± 0.8	65.9 ± 9.8***	1.01 ± 0.13***
Prep. 2			
0	98.0 ± 0.6	2.6 ± 0.9	ND
1	98.1 ± 0.5	1.5 ± 0.9	ND
5	97.6 ± 0.4	1.7 ± 0.7	ND
10	96.5 ± 0.9	3.8 ± 0.6	0.21 ± 0.30**
40	97.5 ± 0.9	46.9 ± 26.9**	0.59 ± 0.12**
160	99.7 ± 0.7	35.8 ± 5.4*	0.48 ± 0.17*

Table 4 Importance of preincubation with sodium selenite in conferring resistance of HUVEC to oxidative damage induced by 100 μM t-BuOOH

HUVEC were cultured in Se-deficient medium or the same medium supplemented with specified concentrations of sodium selenite until confluent in 12-well plates. LDH retention was measured in response to exposure to 100 μM t-BuOOH for 20 h. In parallel, HUVEC cultured in Se-deficient medium for the same time period were treated for 20 h with a solution of 100 μM t-BuOOH containing the specified concentrations of sodium selenite (no preincubation). All incubations were carried out in triplicate. Significant differences from Se-deficient cells: $^{***}P < 0.001$.

	LDH retention (%)			
	Preincubation		No preincubation	
Sodium selenite (nM)	0 μM t-BuOOH	100 μM t-BuOOH	0 μM t-BuOOH	100 μM t-BuOOH
0	90.6 ± 1.4	8.1 ± 0.7	90.2 ± 1.4	8.0 ± 1.0
5	91.7 ± 1.5	6.9 ± 0.8	92.9 ± 1.1	7.6 ± 1.0
10	91.9 ± 0.8	8.4 ± 0.3	92.3 ± 0.4	11.1 ± 0.8
40	97.6 ± 0.9	89.7 ± 0.6***	91.4 ± 0.22	9.7 ± 0.3
160	99.7 ± 0.7	87.2 ± 1.1***	91.9 ± 0.9	7.7 ± 0.7

effect was observed when selenite and t-BuOOH were added simultaneously to cells grown in Se-deficient medium (Table 4).

Selenoprotein expression in EC in response to sodium selenite

In all of the EC preparations, selenite added at 5–10 nM produced a significant ($P < 0.05$) increase in the activity of TR, and 40 nM selenite resulted in maximal induction of TR activity (Tables 1–3). The concentration of TR was also increased maximally in the presence of 40 nM selenite in the human EC (Tables 1 and 2). It was not possible to determine TR concentration in the BAEC, because bovine TR showed no cross-reactivity with the anti-(human TR) antibody. In BAEC grown in Se-deficient medium, we were unable to detect TR activity.

In the presence of > 10 nM selenite, TR activity was measurable, with maximal activity found in the presence of 40 nM selenite. The TR activity in Se-supplemented BAEC was > 25-fold lower than the activity in HUVEC or HCAEC.

Due to the limited number of cells, it was not possible to measure GPX-1 and GPX-4 activities in each of the preparations. In those preparations where these activities were determined, maximal induction was produced by 40 nM selenite. The mean inductions of TR activity, TR concentration, GPX-1 activity and GPX-4 activity in the five human EC preparations, expressed as multiples of the basal value, were (means ± S.D.) 3.76 ± 1.01, 2.18 ± 0.87, 3.38 ± 1.86 and 2.54 ± 0.36 respectively. The induction of TR activity in response to selenite was significantly ($P < 0.05$) greater than the induction found in TR concentration. The activities of GPX-1 and GPX-4 in HUVEC grown in medium containing 40 nM Se (as selenite) were 0.086 ± 0.024 units/mg of protein and 7.147 ± 2.93 m-units/mg of protein respectively; these values were not significantly different from the activities of these selenoperoxidases found in BAEC (GPX-1, 0.115 ± 0.007 units/mg of protein; GPX-4, 4.54 ± 0.76 m-units/mg of protein).

DISCUSSION

The numbers of experiments reported here are small. However, the results establish clearly that HUVEC and HCAEC cultured in Se-deficient medium can be completely protected from the toxic effects of 100 μM t-BuOOH by the addition of Se (as selenite) at concentrations as low as 5–40 nM (Tables 1 and 2). Selenite added in the absence of t-BuOOH had no significant effect on LDH retention (Tables 1 and 2). The results also indicate that HCAEC and HUVEC have similar sensitivities to the harmful effects of t-BuOOH, and that the two cell types show similar responses to Se, in terms of the trace element's ability to confer an antioxidant action.

There were small differences in the sensitivity of the various human EC preparations to t-BuOOH toxicity. These differences probably reflect variability in endogenous antioxidant defence mechanisms, such as selenoproteins, catalase and superoxide dismutase, which all work in concert to maintain the cell's redox potential [21,22]. Variable susceptibility to toxic agents between different isolates of HUVEC, and between passage numbers within the same isolate, has been reported in other studies [23].

The ability of EC treated with 40 nM selenite to resist t-BuOOH toxicity is likely to be due to increased selenoprotein expression, since a preincubation period of EC with sodium selenite was necessary to confer protection. Similarly, skin cells in culture can only be protected from the lethal effects of UVB radiation by selenite if a preincubation step is performed [24].

The modification of selenoprotein activity in response to Se supplementation in cultured cells has been widely reported. For example, Se supplementation has been shown to induce GPX-1 and GPX-4 activities in human and bovine EC [7,25]. In the present study, we have now shown that the activities of GPX-1, GPX-4 and TR are increased in HCAEC and HUVEC by approx. 3-fold in response to selenite supplementation. The increase in TR activity in response to sodium selenite was greater than the corresponding increase in TR concentration, as determined by our RIA that is specific for the cytoplasmic TR isoenzyme. This discrepancy might arise if Se supplementation induces both the cytoplasmic and mitochondrial forms of TR in human EC [26].

The concentration of sodium selenite (40 nM) that led to maximal induction of TR and the GPXs was identical with the selenite concentration that conferred complete protection against t-BuOOH toxicity. It was not possible to consistently find a concentration of selenite that conferred optimal resistance to toxicity and yet produced an increase in the activity of one particular selenoprotein. Thus our results in HUVEC and HCAEC indicate that TR, GPX-1 and GPX-4 are all likely to be involved to some extent in protecting the endothelium from oxidative damage. However, our data suggest that, in human EC (but possibly not BAEC), TR may be a particularly important selenoprotein as regards antioxidant action. This is because (i) human EC contain, on a molar basis, more TR than GPX-4 and GPX-1 [14]; (ii) human EC contain quantitatively more TR than other tissues [14]; (iii) TR is able to detoxify some damaging lipid hydroperoxides more efficiently than GPXs [27]; (iv) TR expression is increased by doses of Se that confer protection from oxidative damage on the EC (Tables 1–3); and (v) BAEC are more sensitive to t-BuOOH than are human EC; these cells have similar activities of GPX-1 and GPX-4, but much lower TR activity (Tables 1–3).

In conclusion, we have shown that small increases in sodium selenite concentration can confer highly significant protection against oxidative damage induced by t-BuOOH in HCAEC and HUVEC. Such protection appears to be related to the increased expression of selenoproteins, rather than to a direct antioxidant effect of sodium selenite. The concentrations of Se required to elicit optimal antioxidant protection are similar to the concentrations required to maximally induce the activities of TR, GPX-1 and GPX-4 in human EC, suggesting that each of these selenoproteins has important antioxidant actions. However, the very high expression of TR in HUVEC and HCAEC, but not in bovine EC, might indicate that TR may be a particularly effective antioxidant selenoprotein in humans. Our data are also consistent with epidemiological evidence indi-

cating that low Se status predisposes to endothelial injury and atherosclerosis.

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SELENIUM PROTECTS KERATINOCYTES FROM BY ULTRAVIOLET RADIATION-INDUCED LIPID PEROXIDATION AND CELL DEATH BY OXIDATIVE STRESS

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Selenium has been shown to be a photoprotectant for skin, protecting skin cells from cell death, oxidative DNA damage, ultraviolet radiation B (UVB)-induced cytokine induction and from skin cancer in mice. We sought to determine whether inorganic selenium as selenite (SS) or as the organic form selenomethionine (SM) could protect the keratinocyte cell line HaCaT from lipid peroxidation and oxidative stress.

HaCaTs were irradiated with 1000J/m^2 of UVB from Philips TL12 lamps and the cells collected and homogenised at various times after UVB. Malondiadehyde (MDA) is a product of lipid peroxidation brought about by reaction of membrane lipids with reactive oxygen species (ROS). The MDA content of cell homogenates was assayed spectrophotometrically and found to increase 2-4 –fold, 24 h after irradiation. Cells were pre-incubated for 24 h before UVB with 1 nM- 50 nM SS or with 50 nM-200 nM SM. The MDA content in unirradiated cells was 0.31± 0.1 μM; this increased to 1.56 ±0.30μM. 24 h after UVB. Pre-incubation with all concentrations of SS or SM decreased the UVB-induced MDA content by 49 %- 69 % ($p<0.05$, 2 experiments).

Menadione (ME) kills cells by causing lipid peroxidation, DNA strand breaks and other ROS mediated damage,. Both SS and SM dose-dependently protected HaCaTs against cell damage measured by lactate dehydrogenase release) after treatment with ME. ME toxicity was cell density-dependent: cells 50 % confluent showed an ID_{50} of 10.5 μM, cells 100 % confluent had an ID_{50} of 45 μM and cells 2 days post confluent, ID_{50}= 68 μM. Thus selenium protects keratinocytes from damage by reactive oxygen species.

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MECHANISMS OF SELENIUM –INDUCED PROTECTION OF KERATINOCYTES FROM CHEMICAL AND ULTRAVIOLET RADIATION-INDUCED CELL DEATH. M. Lewin[1], R.C. McKenzie[2], A.F. Howie[1], F. Nicol[3], J.R. Arthur[3], G.J. Beckett[1], Departments of Clinical Biochemistry[1] and Dermatology[2], University of Edinburgh and the [3]Rowett Research Institute, Bucksburn, Aberdeen

Selenium (Se) protect keratinocytes (KC) from death induced by 960 J/m^2 of ultraviolet radiation B (UVB) or from chemical agents that induce oxidative stress such as menadione (ME). In both KC and HaCaT cells, Se is protective at 1 nM-100nM. At higher levels protection from UVB damage is lost, although protection from ME-induced damage is maintained. Se exerts its actions through modification of selenoprotein expression. Although the function of many of these Se-containing proteins is unknown, the thioredoxin reductase (TR) and the glutathione peroxidase (GPx) families have important antioxidant roles. We have investigated the role of these selenoproteins in the protection of HaCaTs from UVB- and oxidative- induced cell death.

HaCaTs in culture were supplemented with 0 nM to 1000 nM selenite and TR, cytoplasmic GPx (cGPx) and phospholipid GPx (PLGPx) activity measured as a function of [Se]. TR and PLGPx activities increased 2.2-fold and 1.5-fold respectively with increasing [Se] up to 50 nM, ($p<0.05, n=6$) thereafter they plateaued. However cGPx activity increased 5-fold to a maximum at 50 nM [and decreased thereafter to basal, at 1000 nM ($p<0.001$, $n=6$). This corresponds to the [Se] at which protection is lost in cell survival assays, suggesting an important role for cGPx in Se-mediated protection. In contrast TR appears to mediate protection from ME-induced cell death. Pre-incubation of HaCaTs with gold thioglucose (GT), (10 μ M for 48 h) diminished TR activity by 82% ($p<0.001$, $n=6$) and PLGPx by 29% ($n=6$), but did not inhibit cGPx. Cells were more susceptible to ME (20μM- 60μM) than control cells ($p<0.001$) suggesting a role for TR also in protection of HaCaTs from ME-induced oxidative stress. TR inhibition with GT however, did not affect cell survival following UVB. Thus, different selenoenzymes mediate protection of keratinocytes from UVB and oxidative-induced cell death.